# TESTICULAR FUNCTION IN NORMAL AND POOR SEMEN QUALITY STALLIONS

A Thesis

by

TINA MICHELLE BRYAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2004

Major Subject: Physiology of Reproduction

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Approved as to style and content by:

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# ABSTRACT

Testicular Function in Normal and Poor Semen Quality Stallions. (December 2004)

Tina Michelle Bryan, B. S., Texas A&M University

Chair of Advisory Committee: Dr. Thomas H. Welsh, Jr.

The chromosomal location of endocrine genes was established, and relationships between expression of specific endocrine genes and measures of testis function in normal and poor semen quality stallions was assessed. Consensus primer sequences for glucocorticoid receptor (GR) and luteinizing hormone receptor (LHR) were used to screen the CHORI-241 equine bacterial artificial chromosome (BAC) library. The identity of PCR-positive BAC clones was confirmed by sequencing. Verified BACs were mapped to horse metaphase chromosome spreads by fluorescence *in situ* hybridization (FISH). The BACs containing the GR and LHR were localized by FISH to ECA 14q16-q21 and ECA15q22-q23, respectively. In addition to FISH mapping, the 5000rad horse x hamster radiation hybrid (RH) panel was screened in duplicate. Two-point linkage analysis placed GR 0 cR from LEX047, while LHR was 36.67 cR from TKY011 on ECA14 and ECA15, respectively. Total testicular parenchymal weight, mean daily sperm production (DSP) per gram parenchyma and mean apoptotic rate (406.05 ± 24.33g vs. 180.01 ± 34.41g, 15.29 ± 0.87 vs. 10.24 ± 1.10, 6.70 ± 0.88 vs. 14.25 ± 1.11, respectively) differed ($P<0.05$) between normal (n=8) and poor semen quality (n=5) stallions. Also, plasma estradiol and inhibin concentrations were higher ($P<0.05$) in normal stallions than in poor semen quality stallions. Testicular expression

of estrogen receptor beta (ER beta), βB inhibin, prolactin receptor (PRLR), growth hormone receptor (GHR) and insulin-like growth factor I receptor (IGF-IR) mRNAs were all lower ($P<0.05$) in poor semen quality stallions than in normal stallions.

The BACs and primers developed in this study will facilitate future investigations of GR and LHR gene structure in the horse as well as providing a resource for physiological investigation of these two genes that are primary regulators of stress responsiveness and fertility. These data add important endocrine genes to the horse cytogenetic map. Also, important hormonal and gene expression changes have been identified in poor semen quality stallions for further investigation.

## DEDICATION

This thesis is dedicated to my family for encouraging me and supporting me throughout the process of getting my degree. My wonderful daughter, Sidney, was my inspiration to pursue a better education. Because of my parents and their encouragement, I knew I could do anything. Because of my husband, who is always encouraging and supporting me, I will finally be finished. Last, but most importantly, I thank the Lord Jesus Christ who has carried me through the past four years and brought me to the place where He wants me to be.

## ACKNOWLEDGMENTS

I want to thank my committee members for their support and assistance in completing my research and obtaining this degree. Thank you Dr. Welsh for giving me the opportunity to pursue my education and for encouraging me.

I would like to thank Terry Blanchard and Steve Brinsko for the use of the stallions in this study and the semen quality information. Also, I need to thank Noah Heninger for his assistance and information on the apoptosis in this thesis.

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# CHAPTER I

## INTRODUCTION

The horse industry in the United States, especially in the state of Texas, has a substantial economic impact. Since many horses in Texas are used for breeding purposes, reduced fertility of stallions has a significant adverse economic impact. Subfertility is a relatively common condition in stallions. Although many cases are detected by reduced semen quality, the specific cellular/molecular basis for the altered testicular function is usually unidentified making precise treatment impossible. Research information is needed for identification and treatment of stallion infertility and poor semen quality.

Spermatogenesis and steroidogenesis are critical for fertility of the male animals. Breeding soundness exams (BSE) can be used to identify fertility problems associated with reproductive organs, mating, sexual behavior and ejaculate quality of stallions. However, testing the endocrine system and cell signaling pathways involved in fertility and testicular function is not as simple. Due to the large number of steroids, proteins and paracrine/autocrine factors involved in normal spermatogenic and steroidogenic activity, there are many areas where fertility problems can occurr. Environmental factors, animal handling and stressors also have a large impact on fertility. Therefore, identification of predictable measures of animal fertility is critical in the horse industry. Further research involving cellular/molecular mechanisms would increase our

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This thesis follows the style and form of the Journal of Animal Science.

knowledge about causes of poor semen quality and would help reduce economic losses associated with breeding stallions.

The objectives of this project were to investigate relationships between:

1) seminal parameters

2) testicular germ cell apoptosis

3) circulating hormone concentrations

4) gene expression in the testis

5) *in vitro* testis production of testosterone and StAR protein synthesis

Additionally, our objectives involve tool development for studying stallion cellular/molecular signaling in the testis and mapping important endocrine genes in horses. These objectives will help to identify parameters useful for future research in understanding stallion fertility.

# CHAPTER II

# LITERATURE REVIEW

Horses represent a significant industry in the United States and have a substantial economic impact in the state of Texas. Since many horses in Texas are used for breeding purposes, reduced fertility of mares and stallions has a significant adverse economic impact on the horse industry. The impact of stallion subfertility on economic loss is magnified over that of mares since stallions may be bred to numerous mares during a breeding season. Subfertility is a relatively common condition in stallions. Although many cases are detected by reduced semen quality, the specific cellular/molecular basis for the altered testicular function is usually unidentified making precise treatment impossible. Due to high individual animal value, studying the molecular basis for altered testicular function is quite difficult. However, critical research information is needed for identification and treatment of stallion infertility and poor semen quality.

## Male Spermatogenesis and Steroidogenesis

The male testes have two major functions, which include production of sperm through the process of spermatogenesis and production of male sex hormones through steroidogenesis. Spermatogenesis and steroidogenesis are both necessary for fertility of the animal. Breeding soundness exams (BSE) are used to examine reproductive organs, behavior, quantity and quality of ejaculate, and function of the reproductive endocrine system (Juhasz et al., 2000). Due to the high individual value of stallions and the relatively high incidence of fertility disorders, there is interest in detection methods for

fertility parameters (Juhasz et al., 2000; Varner et al., 2000). Testicular parameters such as daily sperm production (DSP) and DSP per gram of testicular parenchyma, referred to as spermatogenic efficiency, are two of the many parameters used to quantitate sperm production in the stallion (Senger, 1999). Hormone measurements are also used to evaluate the function of the endocrine system of an animal. Measuring baseline concentrations of reproductive hormones are sometimes useful for identifying hormonal imbalances that could be associated with subfertility (Juhasz et al., 2000).

Normal spermatogenesis is a relatively inefficient process that results in the loss of a large number of spermatozoa which do not mature in the adult testis (Clermont, 1962; Huckins, 1978). Apoptosis or programmed cell death has been implicated in the testis as an essential mechanism for removal of developing germ cells from the seminiferous epithelium (Knudson et al., 1995; Rodriguez et al., 1997; Russell et al., 2002) and has recently been reported in the testis of normal stallions (Heninger et al., 2004). Since basal levels of germ cell apoptosis have been identified for stallions with normal testes and semen quality, further research evaluating the role of apoptosis in stallions with poor semen quality is possible and would add useful information for the understanding of molecular mechanisms in the testis.

The major factors that control the production of testosterone by the Leydig cells and spermatozoa by the seminiferous tubules are the gonadotrophic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Figure 1). These two hormones are secreted by the anterior pituitary gland under the control of the pulsatile secretion of gonadotropin-releasing hormone (GnRH) into the pituitary portal vessels.

LH binds to the luteinizing hormone receptor (LHR) to stimulate testosterone production and FSH binds the follicle-stimulating hormone receptor (FSHR) to stimulate spermatogenesis in the testis. Secretion of LH and FSH are also controlled by negative feedback messages from the testis through the secretion of testosterone and inhibin, respectively.

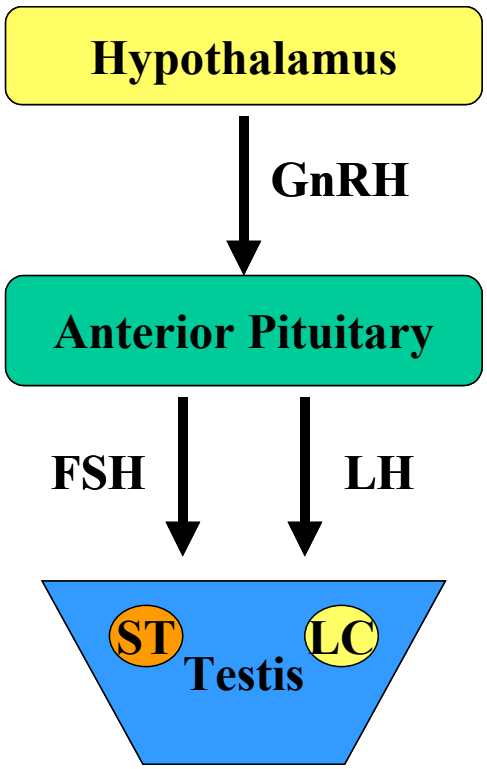


Figure 1. Schematic diagram of the hypothalamic-pituitary-testicular (HPT) axis.

Normal spermatogenesis is dependent upon a functional hypothalamic-pituitary-testicular (HPT) axis, which involves classic endocrine actions of gonadotropins, feedback mechanisms of steroids and proteins and paracrine/autocrine modulation (Roser, 2001). This includes such hormones as LH, FSH, testosterone, estradiol, inhibin and IGF-I as well as many others. Via a negative feedback mechanism, testosterone controls secretion of GnRH, LH and FSH (De Kretser et al., 1995) while supporting spermatogenesis and libido. Also, testicular estrogen appears to play a role in reproductive function in the stallion (Thompson and Honey, 1984; Muyan et al., 1993). Numerous studies have demonstrated that testosterone and estradiol exert negative feedback to inhibit LH secretion and inhibin acts negatively to inhibit FSH secretion (De Kretser et al., 1995). Since all of these hormones create homeostasis in the testis for spermatogenesis, information is needed about hormonal changes in poor semen quality. Plasma inhibin appears to be a useful indicator of reproductive activity in stallions due to a positive correlation between plasma immunoreactive (ir)-inhibin and testicular activity (Nagata et al., 1998a). Motton and Roser (1997) indicate that plasma LH, FSH, estradiol and testosterone concentrations are not different between normal and poor semen quality stallions, but that LH and FSH were significantly higher and estradiol, testosterone and inhibin concentrations were significantly lower in innormal stallions as compared to normal and poor semen quality stallions. Also, Hess and Roser (2001) state that plasma IGF-I concentration does not have a direct relationship with declined fertility in stallions. Since hormone measurements are a non-invasive test method for fertility and these data were from a small population of stallions, further research is warranted to confirm the

results cited in the literature and to examine hormonal relationships and testicular function in the stallion.

### *In Vitro* Testicular Culture Systems

The use of tissue culture methodology has facilitated the understanding of inter-cellular communication as well as synthesis and secretion of many cellular products. Also, culture methods are useful to test specific mechanisms in a more controlled atmosphere than in the animal where environment and stressors can impact the results.

The protein kinase A (PKA) system is considered a cAMP-dependent pathway and works by using three components: 1) receptor, 2) a regulatory component (G protein) and 3) a catalytic component (adenylate cyclase). In the PKA system, binding of the membrane-bound receptor stimulates the regulatory component, also called the G protein and the G protein can be stimulatory (Gs) or inhibitory (Gi). Stimulation of the Gs protein is responsible for activation of adenylate cyclase whereby the biological action of the hormone is initiated. Human chorionic gonadotropin (hCG), produced by the chorion of pregnant women, has an amino acid composition and bioactivity very similar to that of LH and is free of FSH contamination. Therefore, hCG is widely used for research purposes as a substitute for LH, which uses the PKA system for biological action. Forskolin, a chemical isolated from the roots of the Coleus plant, has proven to be a reliable tool for the study of second messenger systems. Forskolin activates adenylate cyclase without the aid of the Gs protein (Seamon and Daly, 1981). Culturing testicular parenchyma allows for the addition of compounds such as hCG, which elicits an LH-like response without affecting FSH activity, and forskolin, which activates

cAMP directly. HCG would enable testing the LHR signaling mechanisms in the stallion testis. Addition of forskolin to cultured testicular parenchyma allows testing of the second messenger system through activation of adenylate cyclase, which bypasses the LH receptor. Both hCG and forskolin use would initiate steroidogenesis in the testicular Leydig cells and stimulate synthesis of testosterone. The testosterone can be assayed by RIA methods to detect changes due to *in vitro* challenges of testis tissue and Leydig cell stimulation. This is a diagnostic tool to directly test the secretory capacity of the Leydig cells (Juhasz et al., 2000). The sites of LH and forskolin action and the pathway of testosterone production are diagramed in Figure 2.

Other changes such as gene expression in the testis can be measured to determine effects of hormone pathways and second messenger systems on male fertility. The biosynthesis of all steroid hormones begins with the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane by StAR protein (Stocco and Clark, 1996). Subsequently, the conversion of cholesterol to pregnenolone by the cytochrome P450 side-chain cleavage (P450scc) enzyme complex takes place and starts the cascade of enzymatic reactions required for testosterone production (Kerban et al., 1999). Previous studies have demonstrated regulation of StAR mRNA with hCG treatment (Kerban et al., 1999) which suggests that hCG challenges affect gene expression in the testis. Changes in StAR and P450scc proteins can be measured using western blot techniques to analyze differences in expression due to hCG or forskolin treatment of testis tissue and to examine relationships with level of testicular function.

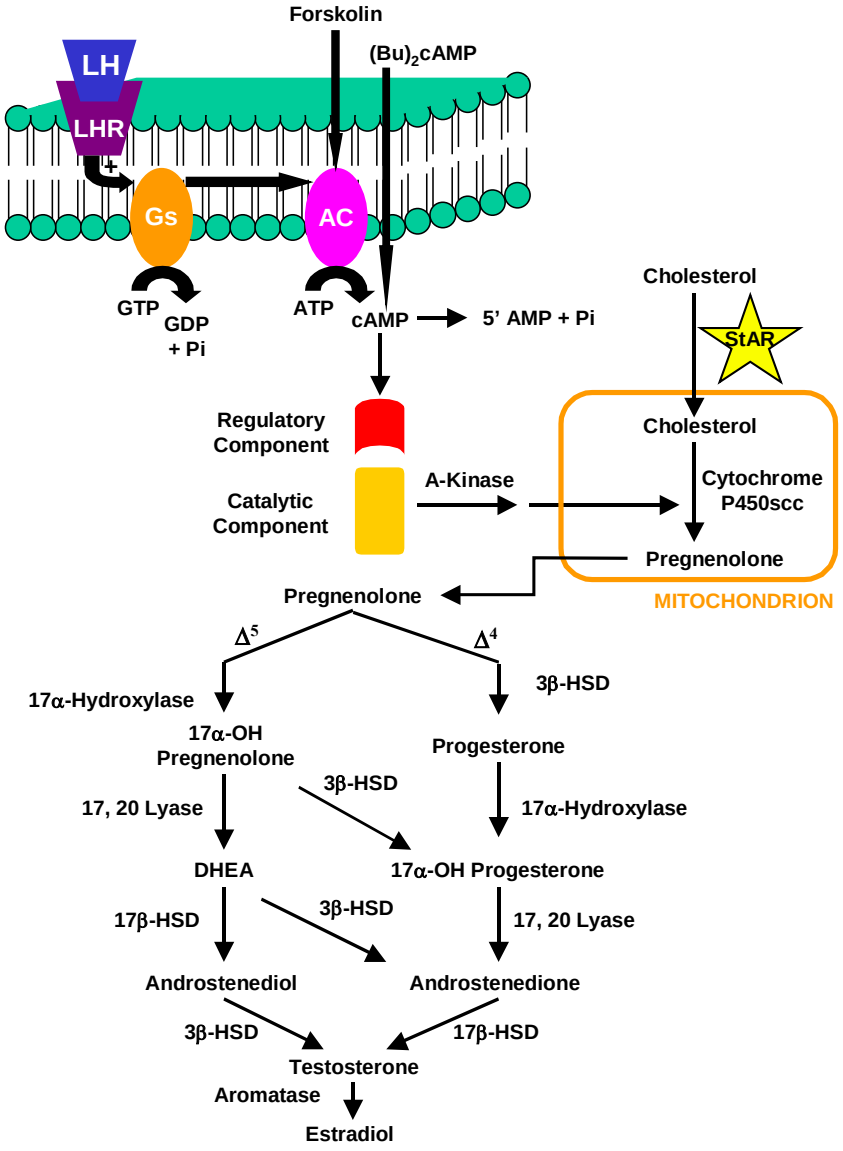


Figure 2. Schematic diagram of the activation of Leydig cell steroidogenesis by luteinizing hormone (LH), forskolin and $(Bu)_2$cAMP. Figure abbreviations are as follows: membrane bound receptor (R), stimulatory G protein (Gs), adenylate cyclase (AC), cyclic AMP (cAMP), protein kinase A (A-Kinase), dihydroepiandrosterone (DHEA) and hydroxysteroid dehydrogenase (HSD). (Modified from French, 1988)

**Gene Expression in the Testis**

The StAR protein mediates the rate-limiting step in steroidogenesis where cholesterol is transported to the cholesterol P450scc enzyme. The expression of StAR protein in the stallion testis is essential for steroid hormone production of testosterone, a hormone essential for libido and fertility. A reduction in StAR gene expression would likely result in a reduction in steroidogenesis (Leers-Sucheta et al., 1999) and a decrease in testosterone production.

Localization of StAR protein has been demonstrated in ovaries of the mare (Watson et al., 2000) but expression patterns of StAR in the stallion testis have not been reported. GR, LHR and StAR gene expression have been demonstrated both in the mouse and rat testis, but have not been studied in the stallion. Controversial results are available for expression patterns of the glucocorticoid receptor (GR) in the testis. Various techniques and authors have demonstrated expression of GR in some cell types of the testis but the results are not in agreement (Weber et al., 2000). By *in situ* hybridization GR is expected to be present in Leydig cells, spermatogonium, macrophages, spermatocytes, round spermatids and sperm. LHR and StAR expression have been demonstrated in the Leydig cells of the testis in the human, mouse, rat, bull and pig (Wahlstrom et al., 1983; Sundby et al., 1984; Pollack et al., 1997; Aspden et al., 1998; Thompson et al., 1999; Garmey et al., 2000; Kotula et al., 2001). Since the Leydig cells are involved in testosterone production in the stallion as in other species, the same expression could be found in the stallion.

Reproductive competence of an individual depends on appropriate expression of the *LHR* gene. The LHR is necessary for steroidogenesis and sperm production in males (Saez, 1994; Ascoli et al., 2002). Endogenous and exogenous glucocorticoids may modulate libido and fertility by inhibition of genes involved in androgen biosynthesis (McKenna et al., 1979; Welsh et al., 1979, 1982; MacAdams et al., 1986). In particular, testicular expression of the GR gene may negatively control expression of the testicular LHR gene.

There are many other genes that have a role in the regulation of steroidogenesis and spermatogenesis. There is a lack of information and understanding of the gene expression patterns in the stallion testis as well as their effect on stallion fertility. Other species are often relied upon as surrogate indicators or predictors of gene expression and function in the stallion. This philosophy is a problem due to the unique development and maturation of the stallion testis in comparison to other species. Studies are needed to determine gene expression and relationships to testicular function. This initial study focused on genes related to steroid and sperm production: namely, we studied a) receptors for classic pituitary-derived factors such as LHR, follicle-stimulating hormone receptor (FSHR), prolactin receptor (PRLR) and growth hormone receptor (GHR); b) steroid receptors such as androgen receptor (AR), estrogen receptor (ER) and glucocorticoid receptor (GR); c) growth factors such as insulin-like growth factors I and II and insulin-like growth factor I receptor (IGF-IR); and d) others such as Steroidogenic acute regulatory (StAR) protein and inhibin.

**Gene Mapping**

This project involved characterizing and gene mapping of the equine glucocorticoid receptor (GR) and luteinizing hormone receptor (LHR). Also, gene expression standards were established for GR, LHR and StAR genes in the normal stallion testis and the developing stallion testis to allow for future research in understanding causes of stallion infertility and poor semen quality problems.

Two isoforms of GR are formed from one gene by alternative splicing. GR alpha has been identified as the active isoform of GR since it binds ligand in the cell cytoplasm and subsequently the complex translocates into the nucleus binding DNA glucocorticoid response elements (GREs) and altering the expression of target genes by repression or activation of gene transcription. The GR beta isoform is present in the nucleus of the cell and does not bind ligand (Pujols et al., 2002; Schaaf and Cidlowski, 2003). The function of GR beta is not fully understood but is thought to bind GR alpha in certain cell types to prevent GR alpha from binding ligand and being activated (Yudt et al., 2003). The overexpression of GR beta is associated with pathophysiologic conditions due to glucocorticoid resistance (Yudt et al., 2003) because neither the exogenous nor endogenous GC can interact with GR alpha due to interference by GR beta.

The GR mediates the anti-inflammatory actions of GCs. Therefore, it is of paramount importance to understand GR gene expression under physiologic and pathophysiologic states (i.e., Cushing's syndrome and glucocorticoid resistance). Intra-synovial administration of GCs (naturally occurring and longer-acting synthetics) is a common treatment for noninfectious inflammatory joint injuries unassociated with bony

or articular fractures. Therapy of acute neurologic disease and ataxia often includes systemic administration of large doses of GCs to relieve inflammation of the spinal cord and meninges. It is important to identify doses that will therapeutically relieve local inflammation, without provoking unwanted side effects on immune and reproductive functions (Rowland et al., 1998; McKay and Cidlowski, 1999; Schmidt et al., 1999) that are manifested by expression of the GR gene and activation of the GR protein.

Gonadal expression of GR mediates the inhibitory effect of exogenous and endogenous glucocorticoids on differentiated reproductive functions. Analogously, adrenal expression of LHR has been linked with development of adrenocortical tumorigenesis and overproduction of cortisol, which is characteristic of Cushing's Syndrome (Bielinska et al., 2003; Feelders et al., 2003; Mikola et al., 2003). The availability of species-specific probes will allow a more detailed molecular definition of the physiologic and pathophysiologic coupling of the adrenal and gonadal axes.

One of our objectives was to have primers that would distinguish between the hormone binding (GR alpha) and non-hormone binding (GR beta) isoforms of GR in the horse. By distinguishing between the isoforms it allows for a better understanding of function as well as confirmation of the presence of both isoforms in the horse. The synteny of GR beta is not conserved across species and is absent in the mouse (Otto et al., 1997). This leads to questions of the physiological significance of the GR beta isoform in other species. Since *GR* mapped to human chromosome 5, our hypothesis was that *GR* would map to either equine chromosome 14 or 21 (Chowdhary et al., 2003).

Compared to the Human Genome Project, limited information is available regarding the Equine Genome. Mining of the Human Genome accelerates the study of endocrine and immune system genes in domestic animals including the horse. Identification of the chromosomal locations of GR and LHR in the horse allows for future research and development of tests for determination of GC excess as in the case of Cushing's syndrome in the horse. Availability of equine specific probes will allow for clarification of LHR role in reproduction and GR isoforms for use in gene expression studies of glucocorticoid resistance.

By using methods such as seminal parameter measurements, germ cell apoptotic rate, circulating hormone concentrations, *in vitro* testicular parenchyma culture techniques and gene expression, the molecular mechanisms of testicular function might be better evaluated. The objectives of this project were to investigate relationships between:

1) seminal parameters

2) testicular germ cell apoptosis

3) circulating hormone concentrations

4) gene expression in the testis

5) *in vitro* testis production of testosterone and StAR protein synthesis

Additionally, our objectives involve tool development for studying stallion cellular/molecular signaling in the testis and mapping important endocrine genes in horses. These objectives will help to identify parameters useful for future research in understanding stallion fertility.

# CHAPTER III

# DEVELOPMENT OF MOLECULAR TOOLS TO STUDY GR, LHR, AND StAR PROTEIN*

## Introduction

Characterization of the equine genome through the construction of dense gene maps will markedly facilitate investigation of physiologic processes. Relative to the mouse, human and bovine genomes, far fewer genes have been positioned on the equine gene map and only a very few of these are associated with endocrine or immune function. The availability of the first generation whole genome radiation hybrid map for the horse is a significant recent advance (Chowdhary et al., 2002). Given the paucity of genes from the endocrine axes that have been mapped in horse, we have focused on loci that will be of use in physiological studies. Herein, we describe the chromosomal localization of nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor; *GR* also known as *NR3C1, GCR or GRL*), luteinizing hormone/choriogonadotropin receptor (*LHR*, also known as *LHCGR, LCGR and LGR2*) and steroidogenic acute regulatory protein (*StAR*). The proteins derived from the *GR*, *LHR* and *StAR* genes are pivotal regulators of adrenal and gonadal function, respectively.

*GR* plays a critical role in mediating physiologic, pathologic and therapeutic actions of glucocorticoids (GCs; Schaaf and Cidlowski, 2002). GCs such as cortisol and

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*Reprinted with permission from "Chromosomal Localization of Equine Glucocortiocid Receptor (*NR3C1*) and Luteinizing Hormone Receptor (*LHCGR*) Genes by Fluorescence *In Situ* Hybridization and Radiation Hybrid Mapping" by T. M. Bryan, 2004. Animal Genetics (accepted).

corticosterone are a vital class of steroid hormones that regulate protein, fat and carbohydrate metabolism as well as homeostasis. GCs are potent suppressors of immune system response and inflammation. Natural and synthetic forms of GCs have been used for over fifty years for therapeutic intervention in a broad range of autoimmune and inflammatory disorders. These biologic actions of GCs are executed via the GR protein thus *GR* gene expression is vital to sustaining health of an individual (Yudt and Cidlowski, 2002).

Reproductive competence of an individual as well as survival of a species depends on appropriate expression of the *LHR* and *StAR* genes. *LHR* is necessary for steroidogenesis in males and females, sperm production in males and ovum maturation in females (Saez, 1994; Ascoli et al., 2002; Saint-Dizier et al., 2003). Endogenous and exogenous GCs may modulate libido and fertility by inhibition of genes involved in androgen biosynthesis (McKenna et al., 1979; Welsh et al., 1979, 1982; MacAdams et al., 1986). In particular, testicular expression of the *GR* gene may negatively control expression of the testicular *LHR* gene. StAR protein mediates the rate-limiting step of steroidogenesis by the transfer of cholesterol from the outer to the inner mitochondrial membrane of the testis (Stocco and Clark, 1996). Therefore, appropriate *StAR* gene expression is critical for the continual production of steroids such as testosterone in the testis.

*GR* has been mapped to human chromosome 5 (Gehring et al., 1985), mouse chromosome 18 (Francke and Gehring, 1980) and rat chromosome 18 (Goldner-Sauve et al., 1991). To date, *GR* has also been identified in the pig and guinea pig but has not

been mapped yet (http://iowa.thearkdb.org/). *LHR* is encoded by a single gene on human chromosome 2p21, mouse chromosome 17, rat chromosome 6 and sheep chromosome 3 (Rousseau-Merck et al., 1990; Ascoli et al., 2002; http://iowa.thearkdb.org/). *StAR* has been mapped to human chromosome 8, mouse chromosome 8, rat chromosome 16 and cattle chromosome 27 (Sugawara et al., 1995; Yuan et al., 1999; Sonstegard et al., 2000; Bonne et al., 2002). Based on these data, we hypothesized that *GR* would map to either equine chromosome 14 or 21, *LHR* would localize to either equine chromosome 15 or 18 and *StAR* would map to equine chromosome 9 (Chowdhary et al., 2003). The objective of this mapping study is to add important endocrine genes to the equine chromosome map. This would allow future genomics investigation of the placement of endocrine genes and inheritance.

**Materials and Methods**

*Primer Design and PCR Conditions*

Consensus primer sequences for *GR* and *LHR* are listed in Table 1. Three primer sets for *GR* were designed in exons 2, 9A and 9B to facilitate future studies on alternative splicing of equine *GR* (Pujols et al., 2002). *GR* primers were designed by alignment of human (Accession X03348), mouse (Accession NW_000134) and rat (Accession NW_043169) genomic vs. mRNA sequences using ClustalX (ClustalX 1.81; http://bess.u-strasbg.fr/BioInfo/ClustalX/Top.html). Likewise, human (Accession S57793), mouse (Accession M81310), rat (Accession L23500), cattle (Accession AF491303) and sheep (Accession L36329) *LHR* sequences were aligned (Figure 3). PCR conditions for each primer pair were optimized using stallion and hamster genomic

DNA as a template. These primers were used subsequently to screen DNA pools from segment 1 of the CHORI-241 equine BAC library and for RH mapping.

*BAC Library Screening*

The Texas A&M University equine BAC library was assembled using the CHORI-241 equine BAC library from Pieter J. de Jong (Oakland, CA). BAC superpools were made by combining DNA from each well of a 384-well plate into a single-plate pool. Then eight single-plate DNA pools were combined to make a superpool (Figure 4). First, 24 superpools of BAC DNA each representing 8x384 plates were screened. Second, the 8 plate pools corresponding to positive superpools were screened (Figure 5). Once a positive plate was identified, row and column pools were prepared as described by Schibler et al. (1998). Briefly, 50 µl aliquots of BAC clones from overnight cultures were pooled by row and column. After centrifugation at 3000g, bacterial pellets were resuspended in 1ml TE with 1µl used directly as template for PCR. Addresses of PCR-positive BACs were denoted as the intersection of rows and columns (Figure 5). PCR products generated from positive BAC clones were confirmed by sequence analysis using BigDye v2 terminator chemistry (Applied Biosystems, Foster City, CA) prior to mapping.

*Fluorescence* In Situ *Hybridization (FISH) Mapping*

Sequence verified BACs were mapped to horse metaphase chromosome spreads using fluorescence *in situ* hybridization (FISH; see Appendix A for protocol), as

Table 1. Primer design, fluorescence *in situ* hybridization and radiation hybrid mapping results for glucocorticoid receptor (*GR*) and luteinizing hormone receptor (*LHR*).

| **Gene (primer)** | **$MgCl_2$ (mM)** | **Annealing Temp.** | **Equine FISH Results** | **HSA** | **Closest RH marker** | **LOD Score** | **Distance (cR)** |
|---|---|---|---|---|---|---|---|
| **Glucocorticoid Receptor alpha (GRa)** (for: 5' CCTTTCTGTGTGCACCTTACC 3'; rev: 5' TCCATCARCATTTCTTTGACC 3') | 2.0 | 51.5°C | ECA 14q16-q21 | HSA5 | LEX047 | 3.0 | 0 |
| **Glucocorticoid Receptor beta (GRb)** (for: 5' GGCTGTATGAAAATACCCTCC 3'; rev: 5' CCATATTTGGCATTGCTGTA 3') | 3.0 | 53.2°C | ECA 14q16-q21 | HSA5 | LEX047 | 3.0 | 0 |
| **Glucocorticoid Receptor exon 2 (*GR*)** (for: 5' GAAAGCATTGCAAACCTCAA 3'; rev: 5' TCTGTTTTCACTTGGGGCA 3') | 3.0 | 60.0°C | ECA 14q16-q21 | HSA5 | LEX047 | 3.0 | 0 |
| **Luteinizing Hormone Receptor (*LHR*)** (for: 5' GCCATAGACTGGCAGACAGG 3'; rev: 5' AGGCAGCYGAGATGGCAAA 3') | 1.5 | 60.0°C | ECA 15q22-q23 | HSA2 | TKY011 | 3.0 | 36.67 |

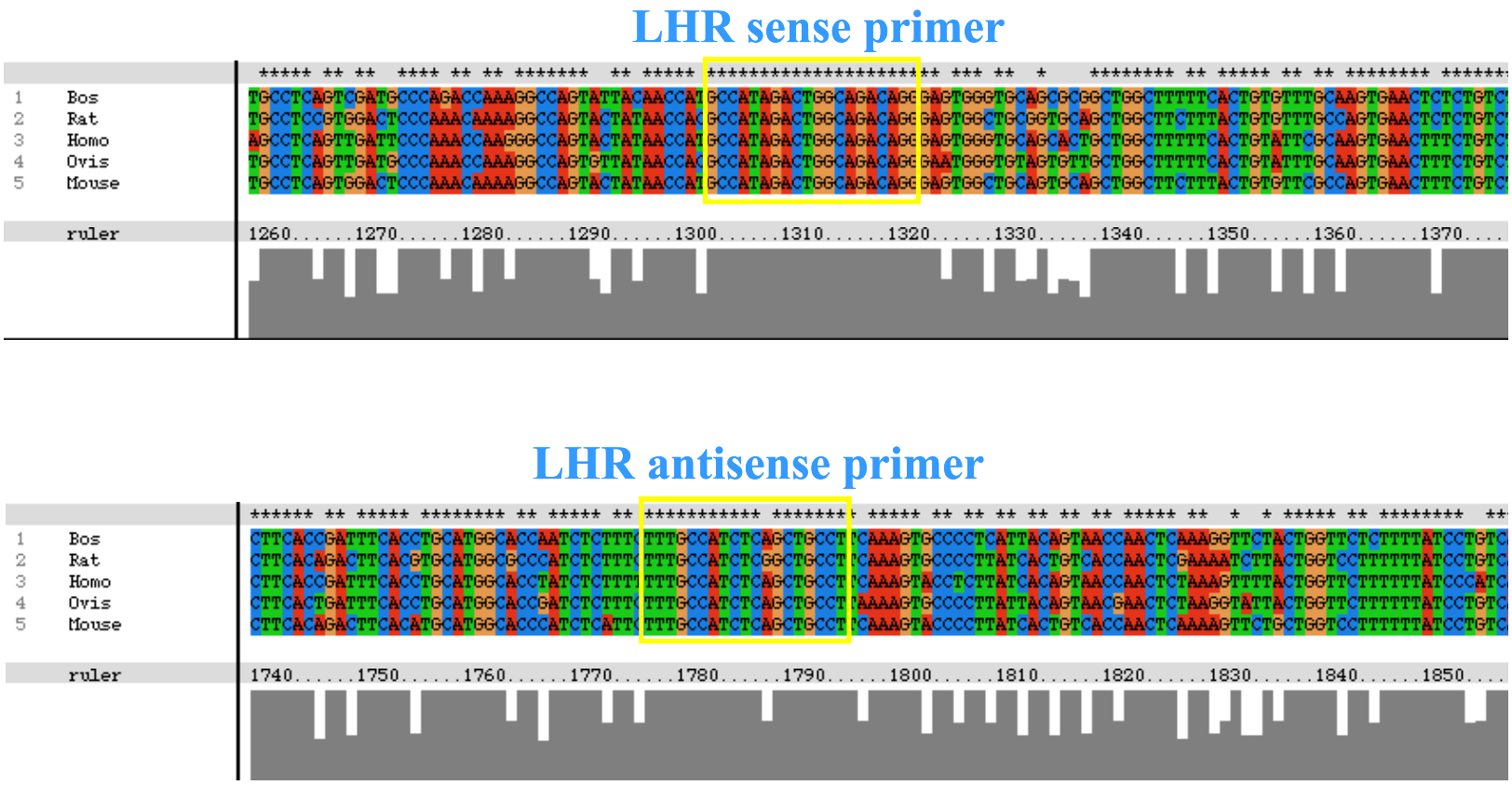


Figure 3. Multiple species sequence alignment of human, mouse, rat, cattle and sheep luteinizing hormone receptor (*LHR*) sequences from GenBank. Sequence similarity was used to identify conserved regions for primer design.

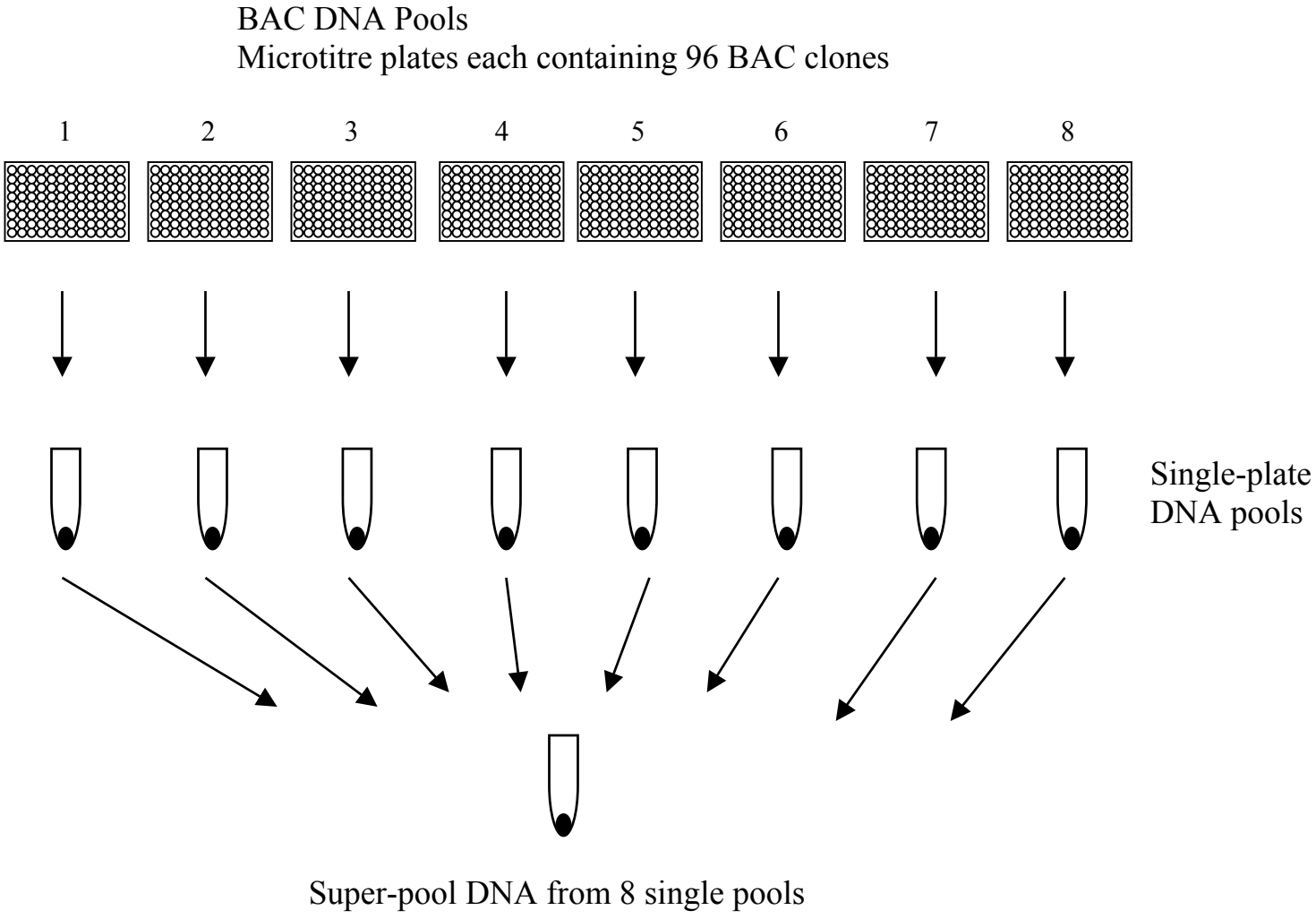


Figure 4. Bacterial artificial chromosome (BAC) clone pooling scheme for the TAMU library.

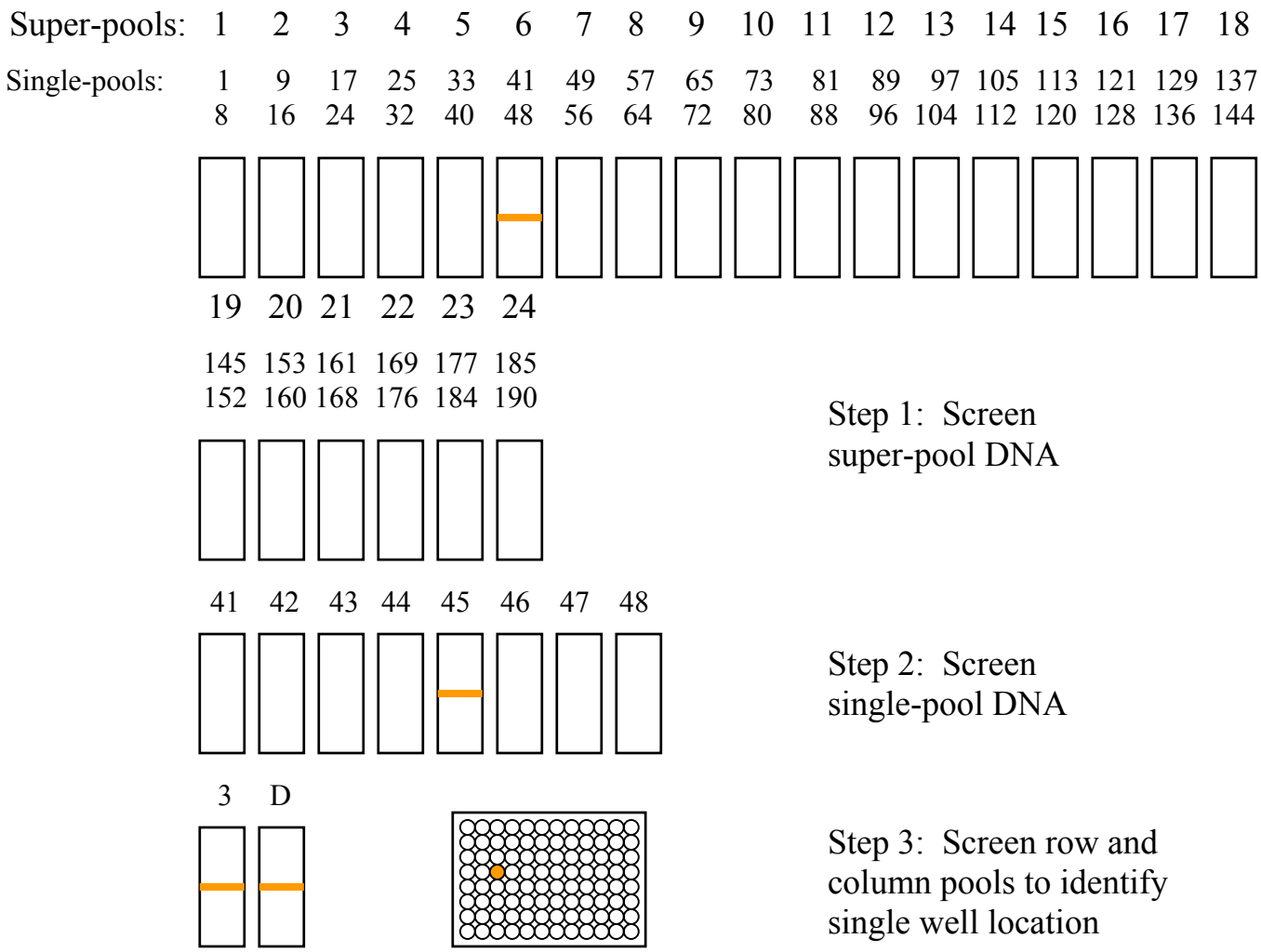


Figure 5. PCR-based method for screening equine BAC library pools. Positive PCR products are shown as gel lanes.

described earlier (Chowdhary et al., 2002). Briefly, ~1ug probes were biotin-labeled by nick translation according to the manufacturer's instructions (BioNick Labeling System, Invitrogen Corp., Carlsbad, CA). The probe was mixed with hybridization solution (2 x SSC, 10% Dextran sulphate & 50% formamide) and applied to metaphase spreads at a final concentration of 20 ng/ul. Details on hybridization, washing and analysis are described elsewhere (Chowdhary et al., 2003).

*Radiation Hybrid (RH) Mapping*

Duplicate RH typing (see Appendix B for protocol) on the 5000rad horse x hamster RH panel was carried out as described by Chowdhary et al. (2002, 2003). Two-point linkage analysis with the markers reported in the first generation RH map (Chowdhary et al., 2003) was performed as per instructions available at http://equine.cvm.tamu.edu/cgi-bin/ecarhmapper.cgi. Marker information, PCR conditions and RH mapping results are summarized in Table 1.

**Results**

*GR* exon 2 primers identified three positive BAC superpools (13, 17, & 24). Singlepools 97, 99 & 131 were identified for further PCR analysis. BACs 097-B1, 099-H1 & 131-C16 were identified as clones containing *GR* exon 2. *GR* exon 9A primers identified two positive BAC superpools (6 & 17). Screening singlepools using exon 9A primers identified one positive (45) for row and column testing. The BAC 045-D15 was identified as a clone containing *GR* exon 9A. *LHR* primers identified two positive BAC superpools (2 & 14). Singlepools 15 and 107 each contained a positive PCR product using *LHR* primers and BAC 015-N8 was confirmed.

PCR products generated using BAC DNA as template were verified by sequencing prior to mapping. The *GR* exon 2 PCR product (Figures 6 and 7) (Accession AY392091, 342 bp: PCR product used for identity comparison with human; Accession AY394747, 461 bp: Cloned product) shared 91% identity with the human genomic sequence for *GR*. A deletion was present in the equine coding sequence eliminating Lys-Leu at amino acid 182-183. Also, there were ten predicted amino acid substitutions in the PCR product for exon 2. The partial sequence for equine *GR* exon 9A (Figures 8 and 9) (Accession AY392092, 330 bp: PCR product used for identity comparison with human; Accession AY550008, 355 bp: Cloning product) shared 97% identity with the corresponding human genomic sequence. A five-nucleotide deletion in the equine sequence was 1288 bases from the human exon 9A stop codon. The equine PCR product (Accession AY392093, 361 bp: PCR product used for identity comparison with human; Accession AY394748, 493 bp: Cloning product) for *LHR* (Figures 10 and 11) corresponded to the human eleventh exon predicted by NCBI Model Maker (http://www.ncbi.nlm.nih.gov/mapview/modelmaker.cgi?contig=NT_022184.13&gene=LHCGR&lid=3973). There was 92% identity between the horse and human genomic sequences. There were eleven predicted amino acid substitutions in the *LHR* PCR product for the horse.

BACs containing *GR* exon 9A and exon 9B were both localized by FISH to ECA 14q16-q21 (Figure 12). RH analysis (Figure 13) assigned the marker to the same chromosome in close vicinity to LEX047 (0 cR distance between the *GR* & LEX047; odds 1:1000 or LOD 3). The location was in agreement with previous observations showing correspondence between ECA14 and HSA5 (Chowdhary et al., 2003).

**Equine GR exon 2 PCR product CHORI_241-131-C16 (342bp) AY392091:**

GCTGTTTCTGCTGCCCCCACAGAGAAGGAGTTTCCAAAAACTCACTCTGAT

GTGTCTTCAGAACAGCAAAATCTGAAGGGCCAGACTGGCACCAGCAGAG

GCAATGTGTATACCACAGACCAAAGAACCTTTGACATTTTGCCGGATTTG

GAGTTTTCTTCTGGGTCCCCAAGTAAAGAGACGAATGAGAGTCCTTGGAG

AGCAGACCTCTTGATAGATGAAAACTGTCTGCTTTCTCCTTTGGCAGGAGA

GGATGATTCATTCCTTTTGGAAGGAAACTCGAATGAAGACTATAAGCCTC

TCGTTTTACCGGATGCTAAACCTAAAATTAAGGATAATGGA

**Translated Equine GR exon 2:**

AVSAAPTEKEFPKTHSDVSSEQQNLKGQTGTSRGNVYTTDQRTFDILPDLEFS

SGSPSKETNESPWRADLLIDENCLLSPLAGEDDSFLLEGNSNEDYKPLVLPDA

KPKIKDNG

Figure 6. Equine glucocorticoid receptor (GR) exon 2 PCR product sequence (AY392091) and translated protein sequence submitted to GenBank.

**Equine GR exon 2 (TA cloned into PCR2.1) (461 bp) AY394747:**

GAAAGCATTGCAAACCTCAATAGGTCGACCAGTGTTCCAGAGAACCCCAA

GAGTTCAGCATCCGTTGCTGTTTCTGCTGCCCCCACAGAGAAGGAGTTTCC

AAAAACTCACTCTGATGTGTCTTCAGAACAGCAAAATCTGAAGGGCCAGA

CTGGCACCAGCAGAGGCAATGTGTATACCACAGACCAAAGAACCTTTGAC

ATTTTGCCGGATTTGGAGTTTTCTTCTGGGTCCCCAAGTAAAGAGACGAAT

GAGAGTCCTTGGAGAGCAGACCTCTTGATAGATGAAAACTGTCTGCTTTCT

CCTTTGGCAGGAGAGGATGATTCATTCCTTTTGGAAGGAAACTCGAATGA

AGACTATAAGCCTCTCGTTTTACCGGATGCTAAACCTAAAATTAAGGATA

ATGGAGATCTGACCTTATCAAGCCCCAACAGTGTGCCACTGCCCCAAGTG

AAAACAGA

**Translated GR exon 2:**

ESIANLNRSTSVPENPKSSASVAVSAAPTEKEFPKTHSDVSSEQQNLKGQTGTS

RGNVYTTDQRTFDILPDLEFSSGSPSKETNESPWRADLLIDENCLLSPLAGEDD

SFLLEGNSNEDYKPLFYRMLNLKLRIMEIPYQAPTVCHCPKKQX

Figure 7. Equine glucocorticoid receptor (GR) exon 2 cloned product sequence (AY394747) and translated protein sequence submitted to GenBank.

**Equine GR exon 9A PCR product CHORI_241-045-D15 (330bp) AY392092:**

ACCTTACCAACTTTCTGTAAACTCAAAACATATTTACTAAGCCACAAGAA

ATTTGATTTCTATTCAAGGTGGCCAAATTATTTGTGTAACAGAAAACTGAA

AATCTAATATTAAAAATATGAAACTTCTAATATATTTTTATATTTAGTTAT

AGTTTCAGATATATATCATATTGGTATTCACTAATCTGGGAAGGGAAGGG

CTACTGCAGCTTTACATGCAATTTATTAAAATGACTGTAAAATAGCTTGTA

TAGTGTAAAATAAGAATGATTTTTAGATGAGATTGTTTTATCATGACATGT

TATATATTTTTTGTAGGGGTCAAAGA

**F**igure 8. Equine glucocorticoid receptor (GR) exon 9A PCR product sequence (AY392092) submitted to GenBank.

**Equine GR alpha (TA cloned in PCR2.1) (355 bp) AY550008:**

CCTTTCTGTGTGCACCTTACCAACTTTCTGTAAACTCAAAACATATTTACT

AAGCCACAAGAAATTTGATTTCTATTCAAGGTGGCCAAATTATTTGTGTAA

CAGAAAACTGAAAATCTAATATTAAAAATATGAAACTTCTAATATATTTTT

ATATTTAGTTATAGTTTCAGATATATATCATATTGGTATTCACTAATCTGG

GAAGGGAAGGGCTACTGCAGCTTTACATGCAATTTATTAAAATGACTGTA

AAATAGCTTGTATAGTGTAAAATAAGAATGATTTTTAGATGAGATTGTTTT

ATCATGACATGTTATATATTTTTTGTAGGGGTCAAAGAAATGCTGATGGA

Figure 9. Equine glucocorticoid receptor (GR) exon 9A cloned product sequence (AY550008) submitted to GenBank.

**Equine LHR PCR product CHORI_241-015-N8 (361 bp) AY392093:**

ACTTTCTGTCTACACCCTCACAGTCATCACACTAGAAAGATGGCACACCAT

CACCTATGCTATTCAGCTGGACCAAAAACTACGATTAAGACATGCCATTC

CAATTATGCTTGGAGGATGGCTCTTTTCTACTCTAATTGCTATGTTGCCCCT

TGTGGGTGTCAGCAATTACAGGAAGGTCAGTATTTGCCTCCCCATGGATAT

AGAAACCACTCTCTCACAAGTCTACATATTAACCATCCTGATACTCAATGT

GGTAGCCTTCTTCATCATTTGTGCTTGCTACATTAACATTTATTTTGCAGTT

CAAAATCCACAACTGATAGCTACCAGCAAAGACACAAAGATTGCTAAGA

AAATG

**Translated Equine LHR:**

LSVYTLTVITLERWHTITYAIQLDQKLRLRHAIPIMLGGWLFSTLIAMLPLVGV

SNYRKVSICLPMDIETTLSQVYILTILILNVVAFFIICACYINIYFAVQNPQLIATS

KDTKIAKKM

Figure 10. Equine luteinizing hormone receptor (LHR) PCR product sequence (AY392093) and translated protein sequence submitted to GenBank.

**Equine LHR (TA cloned in PCR2.1) (493 bp) AY394748:**

AGGCAGCTGAGATGGCAAAAAAAGAGATAGGTGCCATGCAGGTGAAATC

GGTGAAGATGAGGACTGCCATTTTCTTAGCAATCTTTGTGTCTTTGTTGGT

AGCTATCAGTTGTGGATTTTGAACTGCAAAATAAATTTTAATGTAGCAAGC

ACAAATGATGAAGAAGGCTACCACATTGAGTATCAGGATGGTTAATATGT

AGACTTGTGAGAGAGTGGTTTCTATATCCATGGGGAGGCAAATACTGACC

TTCCTGTAATTGCTGACACCCACAAGGGGCAACATAGCAATTAGAGTAGA

AAAGAGCCATCCTCCAAGCATAATTGGAATGGCATGTCTTAATCGTAGTTT

TTGGTCCAGCTGAATAGCATAGGTGATGGTGTGCCATCTTTCTAGTGTGAT

GACTGTGAGGGTGTAGACAGAAAGTTCACTTGCAAATACAGTGAAAAAGC

CAGCAGCACTACACCCACTCCCTGTCTGCCAGTCTATGGC

Figure 11. Equine luteinizing hormone receptor (LHR) cloned product sequence (AY394748) submitted to GenBank.

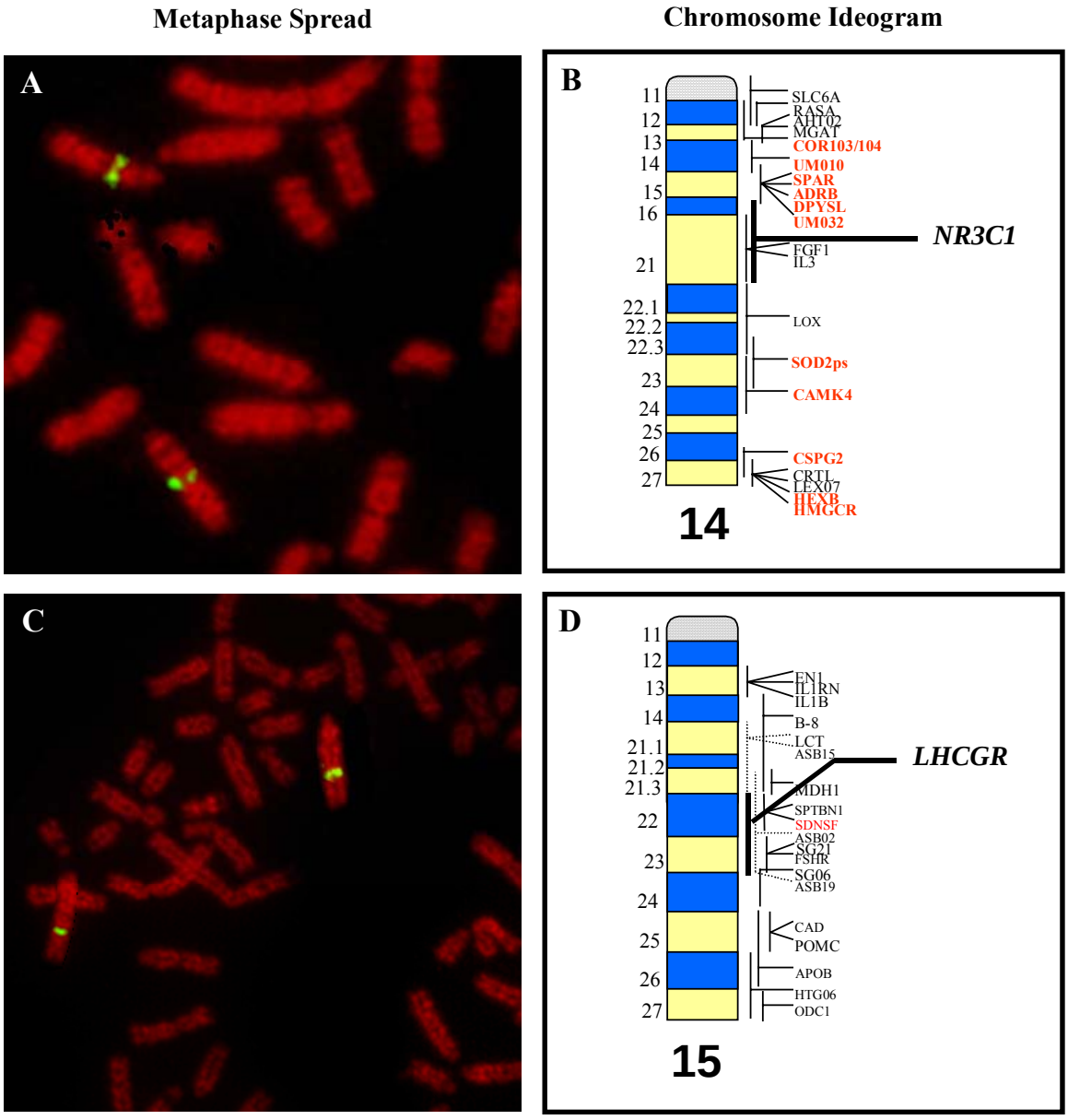


Figure 12. Fluorescence *in situ* hybridization (FISH) with BACs containing the equine glucocorticoid receptor (*NR3C1*) exon 9A (panel A) and luteinizing hormone receptor (*LHCGR*; panel C) on horse metaphase chromosome spreads. G-banded ideograms of ECA14 (panel B) and ECA15 (panel D) show location of the *NR3C1* and *LHCGR* genes, respectively, in relation to the recently published cytogenetic map (Chowdhary et al., 2003).

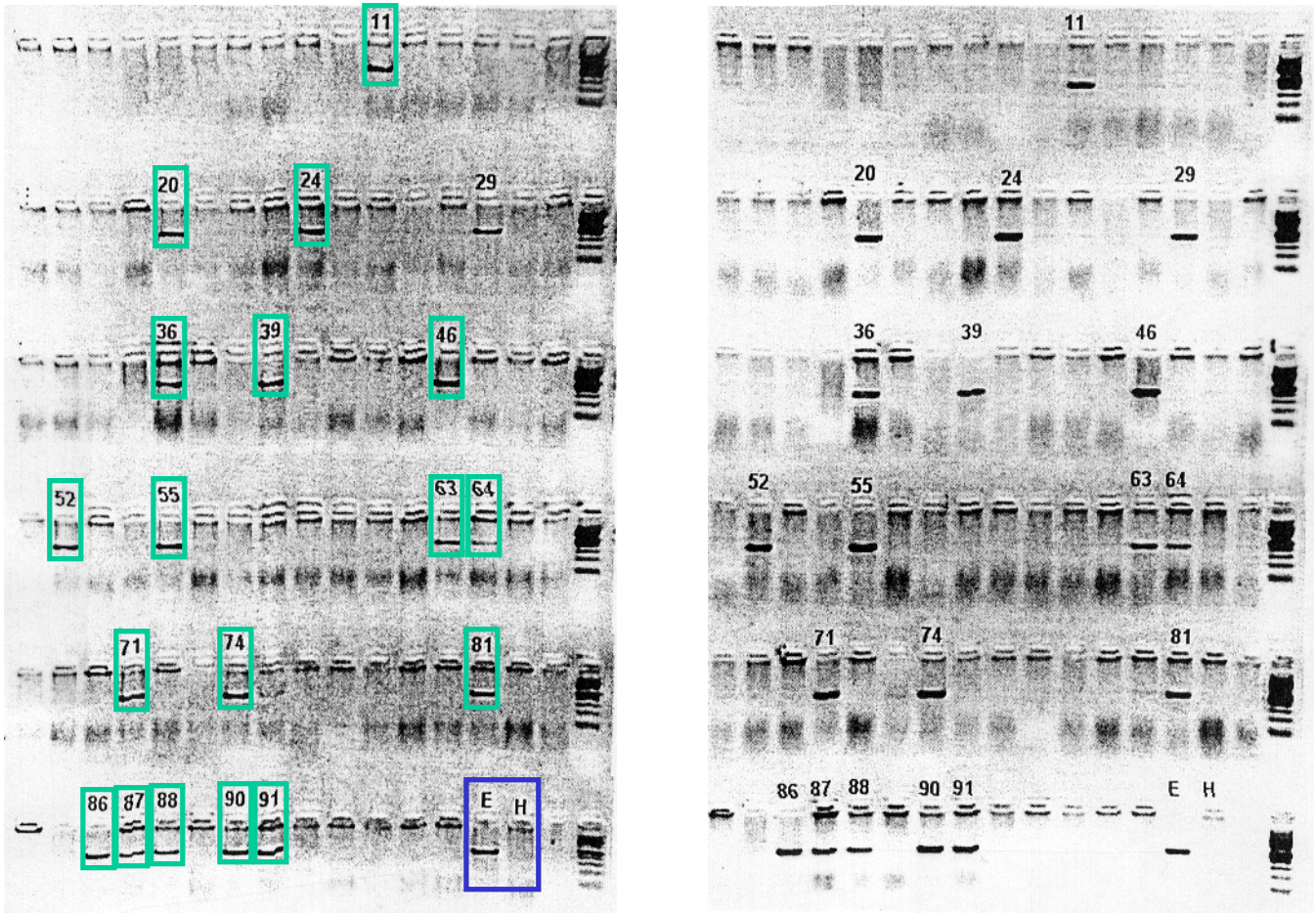


Figure 13. Radiation Hybrid (RH) mapping of the glucocorticoid receptor (GR) exon 2. Two point analysis verifies the presence of *GR* on equine chromosome 14 (ECA14).

The *LHR*-containing BAC was mapped by FISH to ECA15q22-q23 (Figure 12). At odds 1:1000 or LOD 3, RH analysis placed *LHR* at 36.67 cR from TKY011. Our findings concur with earlier observations that indicate homology between ECA15 and HSA2 (Rousseau-Merck et al., 1990; Chowdhary et al., 2003).

The BAC containing StAR protein needs to be isolated from the equine BAC library so that FISH mapping can be performed. This work was planned but due to schedule conflicts and equipment problems the results are still unavailable. Chromosome mapping of StAR protein will be performed in the future using FISH mapping and RH mapping techniques.

**Discussion**

Compared to the investigation of the human genome, limited information is available regarding the equine genome. Mining information from the human genome will accelerate the study of endocrine and immune system genes in domestic animals including the horse. In humans and rats, two isoforms of *GR* are formed from one gene by alternative splicing. These two isoforms share the first eight exons whereas the ninth exon amino acid sequence distinguishes them (Yudt and Cidlowski, 2002). GR alpha expression of target genes is altered by repression or activation of gene transcription. GR alpha has been identified as the active isoform since it binds ligand in the cell cytoplasm, dimerizes and subsequently the complex translocates into the nucleus and binds DNA glucocorticoid response elements (GREs). GR beta is present in the nucleus of the cell and does not bind ligand (Schaaf and Cidlowski, 2003). The function of GR beta is not fully understood but is thought to dimerize with GR alpha in certain cell types to prevent GR alpha from binding ligand and being activated (Yudt et al., 2003). Overexpression of

GR beta is associated with pathophysiologic conditions, which result in glucocorticoid resistance (Yudt et al., 2003) because neither the exogenous nor endogenous GC can interact with GR alpha due to interference by GR beta. Equine GR beta (also known as GR exon 9B) has also been cloned (Figure 14) and the sequence was submitted to GenBank (Accession AY550007, 444 bp: Cloning product) so that further research can be performed to elucidate the function of this GR isoform.

Although the mouse *GR* homolog shares the same gene structure, the alternative splicing event does not occur and GR beta is absent (Otto et al., 1997). This leads to questions of the physiological significance of the GR beta isoform in other species. In this study, BACs that shared sequence similarity with *GR* exons 9A and 9B were recovered and they co-localized to ECA 14q16-21. This suggests that as in other species there is a single *GR* gene and the structural organization of equine *GR* is similar to that of human, rat and mouse. BACs from this study will be useful for further characterization of equine *GR*. Primers were specifically chosen in exons 2, 9A and 9B to facilitate future investigations of alternative splicing of equine *GR* to verify the existence of the GR beta isoform in the horse. Comparison of the equine *GR* sequence to the human *GR* sequence shows a high level of conservation between horse and human. Similarly, equine *LHR* and human *LHR* are highly conserved. The eleventh exon of *LHR* is the gene region involved in activation of G-proteins for signal transduction (Ascoli et al., 2002). Since the equine *LHR* PCR product (AY392093) in our study hybridizes this region of the gene, tools are available to evaluate mRNA expression of *LHR* under physiologic and pathophysiologic conditions.

**Equine GR beta (TA cloned in PCR2.1) (444 bp) AY550007:**

GGCTGTATGAAAATACCCTCCTCAAATAACTTGCTTAACTACATATAGATT

CAAGTGTGTCAATATTCTATTTTGTATATTAAACAAATGCTATATAATGGG

GACAAATCTATATTATACTGTGTATGGATGGCATTATTAAGAAGCTTTTTC

ATTATTTTTTATCACAGTAATTTTAAAATGTGTGAAAATTAAAACCAGTGA

CTCCTGTTTAAAAATAAAAGTTGTAGTTTTTTATTCATGCTAAATAATAAT

CTGTAGTAAAAAAAAAAAAAAAAATGTCTTTTTACCTACGCAGTGAAATG

TCAGATTGTAAAATCTTGTGTGGAAACGTTTAACTTTTATTTTTCATTTAAA

TTTGCTGTTCTGGTATTACCAAACCACACATTTGTACTGAATTGGCAGTAA

ATGTTAGTTAGCCATTTACAGCAATGCCAAATATGG

Figure 14. Equine glucocorticoid receptor (GR) exon 9B cloned product sequence (AY550007) submitted to GenBank.

Two forms of equine *StAR* that only differ in the length of their 3' untranslated region (3' UTR) have been identified (Kerban et al., 1999). The open reading frame (ORF) encodes a 285-amino acid protein that is 86-90% identical to StAR of other species characterized to date (Kerban et al., 1999). *StAR* is a phosphoprotein synthesized in the cytosol as a short-lived 37-kDa precursor that is processed into more stable 30-kDa proteins after mitochondrial import (Epstein and Orme-Johnson, 1991; Stocco and Sodeman, 1991; King et al., 1995). The 37-kDa form of StAR protein is believed to represent the active form of StAR involved in moving cholesterol across mitochondrial membranes for steroid hormone biosynthesis (Stocco and Clark, 1996).

*GR* mediates the anti-inflammatory actions of GCs. Therefore, it is of paramount importance to understand *GR* gene expression under physiologic and pathophysiologic states (i.e., Cushing's syndrome and glucocorticoid resistance). Systemic or intra-synovial administration of GCs (naturally occurring and longer-acting synthetics) is a common treatment for noninfectious inflammatory joint injuries unassociated with bony or articular fractures. Therapy for acute neurologic disease and ataxia often includes systemic administration of large doses of GCs to relieve inflammation of the spinal cord and meninges. It is important to identify doses that will therapeutically relieve local inflammation, without provoking unwanted side-effects on immune and reproductive functions (Rowland et al., 1998; McKay and Cidlowski, 1999; Schmidt et al., 1999) that are manifested by expression of the *GR* gene and activation of the *GR* protein.

Gonadal expression of *GR* mediates the inhibitory effect of exogenous and endogenous glucocorticoids on differentiated reproductive functions. Analogously, adrenal expression of *LHR* has been linked with development of adrenocortical

tumorigenesis and overproduction of cortisol, which is characteristic of Cushing's Syndrome (Bielinska et al., 2003; Feelders et al., 2003; Mikola et al., 2003). The availability of species-specific probes will allow a more detailed molecular definition of the physiologic and pathophysiologic coupling of the adrenal and gonadal axes.

# CHAPTER IV

# GENE EXPRESSION IN THE TESTIS OF NORMAL AND POOR SEMEN QUALITY STALLIONS

## Introduction

Many testicular genes have a role in the regulation of steroidogenesis and spermatogenesis. However, there is a lack of information and understanding of the gene expression patterns in the stallion testis as well as their affect on stallion fertility. Other species are often relied upon as surrogate indicators or predictors of gene expression and function in the stallion. This philosophy is a problem due to the unique development and maturation of the stallion testis in comparison to other species. Studies are needed to determine gene expression, testicular function and fertility of the stallion testis. This initial study focused on genes related to steroid and sperm production: namely, we studied a) receptors for classic pituitary-derived factors such as LHR, follicle-stimulating hormone receptor (FSHR), prolactin receptor (PRLR) and growth hormone receptor (GHR); b) steroid receptors such as androgen receptor (AR), estrogen receptor (ER) and glucocorticoid receptor (GR); c) growth factors such as insulin-like growth factors I and II and insulin-like growth factor I receptor (IGF-IR); and d) others such as the steroidogenic acute regulatory (StAR) protein and inhibin.

The major factors that control the production of testosterone by the Leydig cells and spermatozoa by the seminiferous tubules are the gonadotrophic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These two hormones are secreted by the anterior pituitary gland under the control of pulsatile secretion of gonadotrophin-releasing hormone (GnRH) into the pituitary portal vessels. The secretion

of FSH and LH is also controlled by negative feedback messages from the testis through the secretion of testosterone and inhibin. FSH appears to have a role in the maintenance of spermatogenesis through follicle-stimulating hormone receptors (FSHR) on the seminiferous tubules (Means and Vaitukaitis, 1972), Sertoli cells and spermatogonia (Orth and Christensen, 1977, 1978; Baccetti et al., 1998). LH acts through the presence of luteinizing hormone receptors (LHR) on the surface of Leydig cells (De Kretser et al., 1971) to stimulate the production of testosterone, the androgen that is required for seminiferous tubule function.

Controversy still exists regarding the roles of androgens and estrogens in the testis. Androgens play a crucial role in the development of male reproductive organs and are needed for puberty, male fertility and male sexual function (Dohle et al., 2003). Androgen receptors (AR) have been localized to Sertoli cells, Leydig cells and peritubular cells (Mulder et al., 1975; Sanborn et al., 1977; Verhoeven, 1980; Namiki et al., 1991; Nagata et al., 1998b). Sertoli cell-selective knockout mice have been used to demonstrate that the AR in the Sertoli cell is an absolute requirement for androgen maintenance of complete spermatogenesis (De Gendt et al., 2004). The important role of estrogens in females in physiological and pathological processes is well accepted. Recent evidence demonstrating the importance of estrogens in male reproductive physiology has complicated the division between genders with reference to importance of androgens in males. Both estrogen receptor alpha (ER alpha) and estrogen receptor beta (ER beta) isoforms have been detected in the testis (Denger et al, 2001; Makinen et al., 2001; Mowa and Iwanaga, 2001). ER alpha is localized to Leydig cells, round spermatocytes and spermatids (Pelletier et al., 2000), while ER beta was detected in Sertoli cells and round

spermatocytes (Shughrue et al., 1998). Since ER alpha knockout mice suffer from testicular atrophy and infertility, estrogen is vital for normal testicular function (Mueller and Korach, 2001). While ER alpha knockout mice are completely infertile, ER beta knockout mice appear to heave no compromised fertility (Couse et al., 2001). The importance of ER alpha and ER beta in the testis is still unknown and needs further investigation.

Inhibin is involved in a) the negative feedback mechanism inhibiting secretion of FSH by the anterior pituitary as well as b) paracrine regulation of testicular function (Marchetti et al., 2003). Both inhibin alpha and beta B forms appear to be involved in spermatogenesis and paracrine/autocrine regulation of androgen production (Kaipia et al., 1994; Klaij et al., 1994). Inhibin alpha and beta subunits have been detected in Leydig cells and Sertoli cells of the horse testis (Fujimura et al., 1998; Nagata et al., 1998a). The inhibin beta B appears to be the rate-limiting factor in the production of bioactive inhibin in the testis (Klaij et al., 1992).

There are many other testicular genes that have roles in the regulation of steroidogenesis and spermatogenesis. Steroidogenic acute regulatory (StAR) protein has been detected in the Leydig cells of the testis and is required for the transport of cholesterol from the outer to the inner mitochondrial membrane for steroid biosynthesis (Pollack et al., 1997). The glucocorticoid receptor (GR) has also been identified in Leydig cells, Sertoli cells and germ cells indicating a role in steroidogenesis and spermatogenesis (Weber et al., 2000). The prolactin receptor (PRLR) has been detected in Leydig cells, Sertoli cells and various stages of spermatogenesis (Hondo et al., 1995; Zhang et al., 1995; Jabbour and Lincoln, 1999). The expression of PRLR was

hormonally regulated by inhibiting FSH (Guillaumot and Benahmed, 1999), which indicates a role in spermatogenesis. However, the fertility of PRLR knockout mice was unaffected indicating that the absence of PRL signaling is not detrimental to male testicular function and fertility (Binart et al., 2003).

Growth factors are also thought to play an important role in the paracrine and autocrine control of testicular function (Cailleau et al., 1990; Moore and Morris, 1993; Chandrashekar et al., 2001). Growth hormone receptors (GHR) have been detected in Leydig and Sertoli cells of the testis (Lobie et al., 1990). GHR knockout mice had reduced fertility and altered testicular function (Chandrashekar et al., 1999). Insulin-like growth factor I (IGF-I) was localized in Leydig and Sertoli cells, and in primary spermatocytes, and insulin-like growth factor I receptors (IGF-IR) were found in secondary spermatocytes and spermatids (Vannelli et al., 1988). Insulin-like growth factor II (IGF-II) was localized to Leydig cells in the testis (Koike and Noumura, 1995). These results support paracrine/autocrine roles for growth factors in cell growth, differentiation and development of the testis for steroidogenesis and sperm production.

Only a limited number of studies have been performed to investigate gene expression in the horse testis. The objective of this research was to further investigate relationships between gene expression patterns and testicular function. This initial study focused on genes related to steroid and sperm production: namely, we studied a) receptors for classic pituitary-derived factors such as LHR, FSHR, PRLR and GHR; b) steroid receptors such as AR, ER and GR; c) growth factors such as insulin-like growth factors I and II and IGF-IR; and d) others such as StAR and inhibin.

## Materials and Methods

*Animals*

Testes were surgically removed under general anesthesia from eight mature (ages 4-17 years), light-breed stallions with normal testis size and semen quality and from five mature, light-breed stallions with poor semen quality. The surgical procedure took place at the Texas A&M University Large Animal Clinic. See Table 2 below for stallion identification information and semen quality descriptions.

Table 2. Stallion identification (ID) numbers, classification group and animal problem/diagnosis information for horse samples used in this study.

| Horse ID | Classification Group | Animal Problem/Diagnosis |
|---|---|---|
| 1859 | Normal | |
| 1861 | Normal | |
| 1906 | Normal | |
| 1955 | Normal | |
| 1957 | Normal | |
| 1959 | Normal | |
| 7-5 | Normal | |
| Arab | Normal | |
| Peppy | Poor semen quality | Poor semen quality |
| Leo Castelli | Poor semen quality | Small left testis size |
| King's Travelin Dude | Poor semen quality | No sperm in ejaculate |
| State Dancer | Poor semen quality | Poor semen quality |
| Millenium Impulse | Poor semen quality | Unilateral cryptorchid |

Normal and poor semen quality classification groups were sorted by daily sperm output (DSO). Slices of testicular parenchyma were harvested from the center of the testis

adjacent to the central vein. Several parenchymal samples were snap frozen in liquid nitrogen for RNA analysis and other samples were fixed in 4% paraformaldehyde for *in situ* hybridization.

In Situ *Hybridization (ISH)*

Testis parenchyma samples were placed in 4% paraformaldehyde overnight. After fixation, the samples were processed and embedded in paraffin for cutting sections. *In situ* hybridization (Wilcox, 1993) was used to determine the localization of mRNAs that code for GR alpha, GR beta, GR exon 2, LHR, AR and StAR in the testes (see Appendix C for protocol). Briefly, slides of testis sections were deparaffinized by ethanol washes. Slides were washed with SSC and treated with Proteinase K. Sections were then exposed to radioactively labeled mRNA probes overnight at 55°C for hybridization. After washing slides with SSC and treatment with Rnase A the slides were dehydrated with ethanol and allowed to dry at 37°C. Slides were exposed to X-ray film at room temperature to determine optimal length of exposure to emulsion for each probe. Slides were dipped in emulsion that was diluted 1:1 with water at 42°C and placed at 4°C for 8-10 weeks. Slides were then developed using Kodak D19 developer diluted 1:1 with water (15°C) and Kodak fixer. Toludine blue was used as counter stain of cell nuclei for identification. Slides were cleaned, washed with ethanol and xylene and then cover slipped with Permount (Fisher Scientific). Slides were allowed to dry at least 24 hours before visualization under a microscope. Localization of mRNAs in different cell types of the testis were identified using microscopy for grain visualization and images captured for validation. NIH Image software (National Institutes of Health, Bethesda, MD) was

used for semi-quantitative analysis of *in situ* hybridization to determine the amount of mRNA localization in testis sections.

*RNA Isolation*

At castration, a portion of testicular parenchyma was snap frozen for RNA isolation and analysis. Tripure reagent (Roche, Switzerland) was used to extract mRNA (see Appendix D for protocol) from various stallion tissues such as the adrenal, pituitary, thyroid, liver, spleen and testis. Specifically, 0.3g of tissue was placed in 3 mL of Tripure reagent in a 50 mL tube. Each sample was homogenized twice for 30 seconds each. Total cellular RNA was separated from DNA and protein by first mixing the samples with chloroform and then centrifuging the homogenate. The upper aqueous layer of RNA was then transferred to a clean tube and precipitated first with isopropanol and then with 70% ethanol. RNA pellets were resuspended with diethyl pyrocarbonate-treated (DEPC) water and heated at 68°C for 10 minutes. Samples were then aliquoted to use for gel electrophoresis procedures to check mRNA integrity. Spectrophotometry analysis was used to determine concentration of RNA in the samples. Extracted mRNA was kept frozen at –80°C to minimize degradation.

Integrity of extracted mRNA was confirmed using gel electrophoresis to separate 18S and 28S ribosomal RNA bands (Figure 15). If a sample contained degraded RNA, the sample would appear as a vertical smear for that sample. All of the extracted mRNA samples had acceptable concentrations and RNA integrity. Therefore, the samples were valid specimens for further experimentation and analysis.

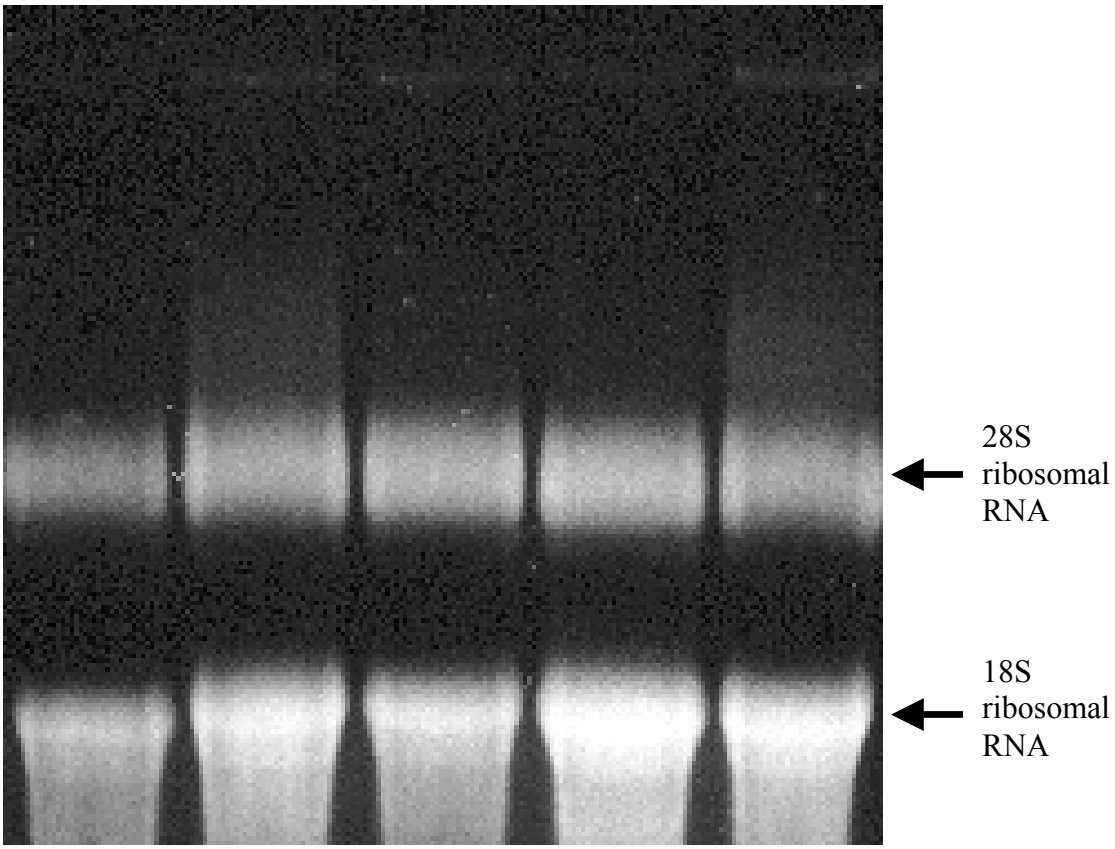


Figure 15. Representative 1% agarose gel image stained with ethidium bromide (EtBr) to allow visualization of 18S and 28S ribosomal RNA bands. If a sample contained degraded RNA, the sample would appear as a vertical smear for that sample. This image is an example of non-degraded samples of RNA for stallion testis parenchyma.

*Probe Validation*

Specificity of newly created probes for GR and LHR were verified using the ribonuclease protection assay procedure (RPA; see Appendix E for protocol). Radioactively labeled GR and LHR probes were synthesized using the Ambion MAXIscript *in vitro* transcription kit (Ambion, Inc., Austin, Texas) where one microgram of cDNA template was transcribed using T7 Polymerase to incorporate $^{32}$P-labeled UTP into the sequence. An RPA was used to visualize and quantitate extracted RNA (described above) of genes of interest following Ambion's RPA III kit (Ambion, Inc., Austin, Texas). Co-precipitation of sample RNA and labeled probe was performed at –20°C for 15 minutes. The RNAs were pelleted by centrifugation and allowed to dry. Pellets were resuspended in 10 µl Hybridization Buffer, incubated at 90°C for 3 minutes and transferred to 42°C overnight. Unprotected single-stranded RNA was digested and protected RNA was precipitated at –20°C. Pellets were resuspended in gel loading buffer and loaded on a 5% denaturing polyacrylamide gel for 2 hours. The gel was exposed to autoradiography film for 24-48 hours for visualization of protected RNA bands.

*Slot Blot mRNA Analysis*

Total cellular RNA was isolated using the previously described method for testis samples from the eight normal and five poor semen quality stallions. The quantity of mRNA in the testis of each stallion was measured on replicate blots using slot blot northern blot techniques (See protocol Appendix J) for 18S ribosomal RNA, follicle stimulating hormone receptor (FHSR), LHR, StAR, GR beta, GR alpha, GR exon 2, prolactin receptor (PRLR), androgen receptor (AR), estrogen receptor alpha (ER alpha), estrogen receptor beta (ER beta), inhibin, insulin-like growth factor I (IGF-I), insulin-like

growth factor II (IGF-II), growth hormone receptor (GHR) and insulin-like growth factor I receptor (IGF-IR). Briefly, 20 µg of mRNA was hybridized to a nylon transfer membrane using a Bio-Rad slot blot apparatus. The mRNA sample was mixed with 20X SSC, formamide and formaldehyde then applied to the membrane using gentle vacuum suction. The membrane was then washed with 10X SSC and allowed to air dry before baking at 80˚C for 2 hours. The membranes were then stored at –20˚C until further use. Next, the membranes were placed in glass roller tubes with the hybridization buffer at 55˚C for 1 hour in a rolling hybridization oven. cRNA probes were synthesized using $^{32}P$ labeled UTP. Unincorporated nucleotides were removed by column purification and counts of radioactivity obtained using a Beckman scintillation counter. One million cpm per mL of cRNA probe was added to each membrane using 18S ribosomal RNA as a control on another replicate membrane. Probes were allowed to hybridize overnight at 55˚C. The next day the radioactive waste was poured in an appropriately labeled liquid waste container and the membranes were washed with a series of SSC/SDS washes at various temperatures to reduce non-specific binding of the probes to the membranes. Membranes were wrapped in Saran™ wrap and exposed overnight using the Typhoon 8600 scanner and variable mode imager. Numerical results were obtained using ImageQuant analysis software. The PROC GLM and least squares means analysis of SAS were used to analyze density of mRNA pixels with 18S ribosomal RNA density as a covariate for each sample.

**Results**

Radioactively labeled $^{35}S$ probes were hybridized to slides containing stallion testis cross-sections. Upon microscopic examination of the slides, the ISH silver grains

only faintly appeared for GR alpha, GR beta and LHR thus signal strength appears the same as background. The ISH procedures need to be repeated for these genes and slides exposed for longer than ten weeks in order to be able to visualize silver grains and determine localization of mRNAs. The low signal intensity of GR and LHR is expected since the expression of these steroid receptors is low in the testis. A difference in signal above background is subjectively detectable with GR exon 2 and AR by microscopic visualization (Figures 16 and 17). GR exon 2 mRNA localized in a general pattern over the interstitial space and within the seminiferous tubule to cell types throughout the testis. AR mRNA localized primarily to the interstitial space and the perimeter of the seminiferous tubules, but was not present in the center of the seminiferous tubules. This would confirm the presence of AR in Leydig and Sertoli cells but not present in spermatozoa.

StAR protein mRNA localized to Leydig cells in the interstitial space between seminiferous tubules (Figure 18). The quantity of StAR protein mRNA localization was not different between normal and poor semen quality stallion testis cross-sections (36.58 ± 4.91 number of silver grains for normal and 26.84 ± 8.02 number of silver grains for poor semen quality; Figure 19).

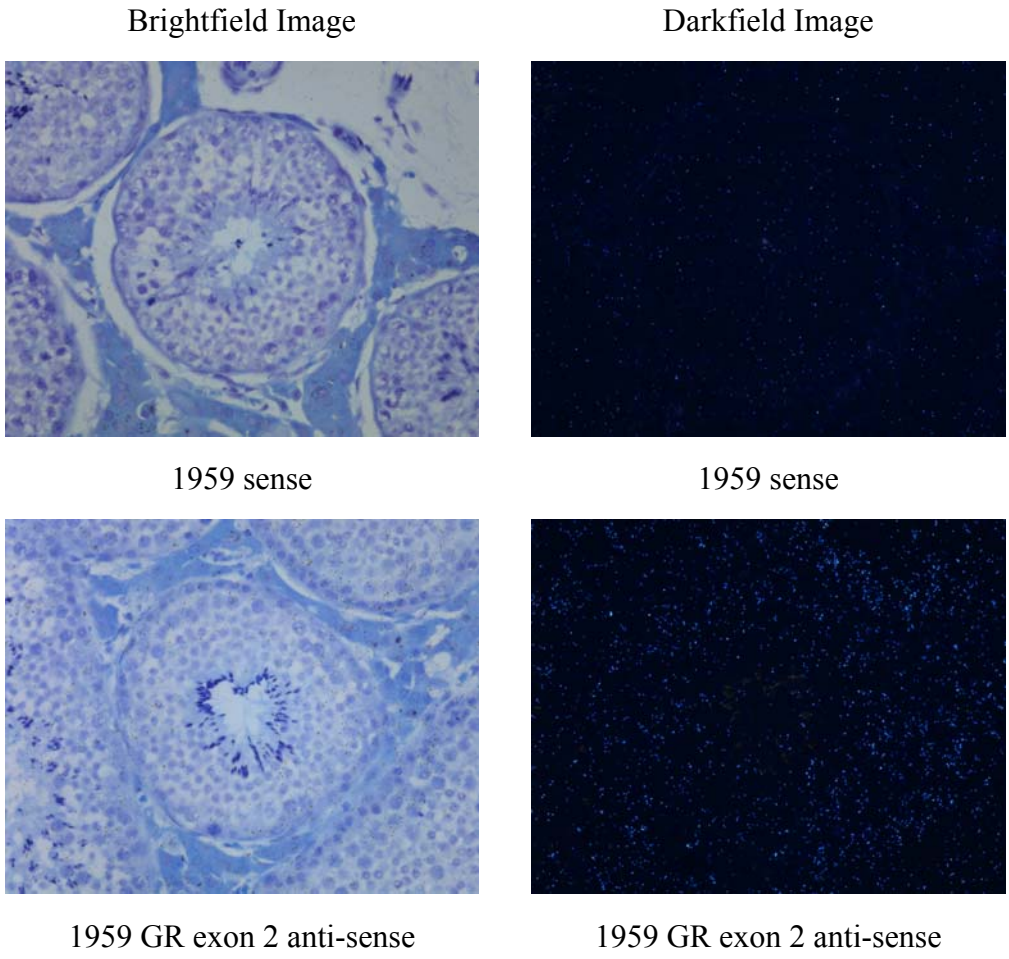


Figure 16. *In situ* localization results for the glucocorticoid receptor exon 2 (GR exon 2) in the stallion testis. GR exon 2 mRNA localized in a general pattern over the interstitial space and within the seminiferous tubule to cell types throughout the testis. $^{35}$S-labeled anti-sense and sense probes were hybridized to testis cross-sections in a normal stallion (animal number 1959). Brightfield and darkfield images are displayed.

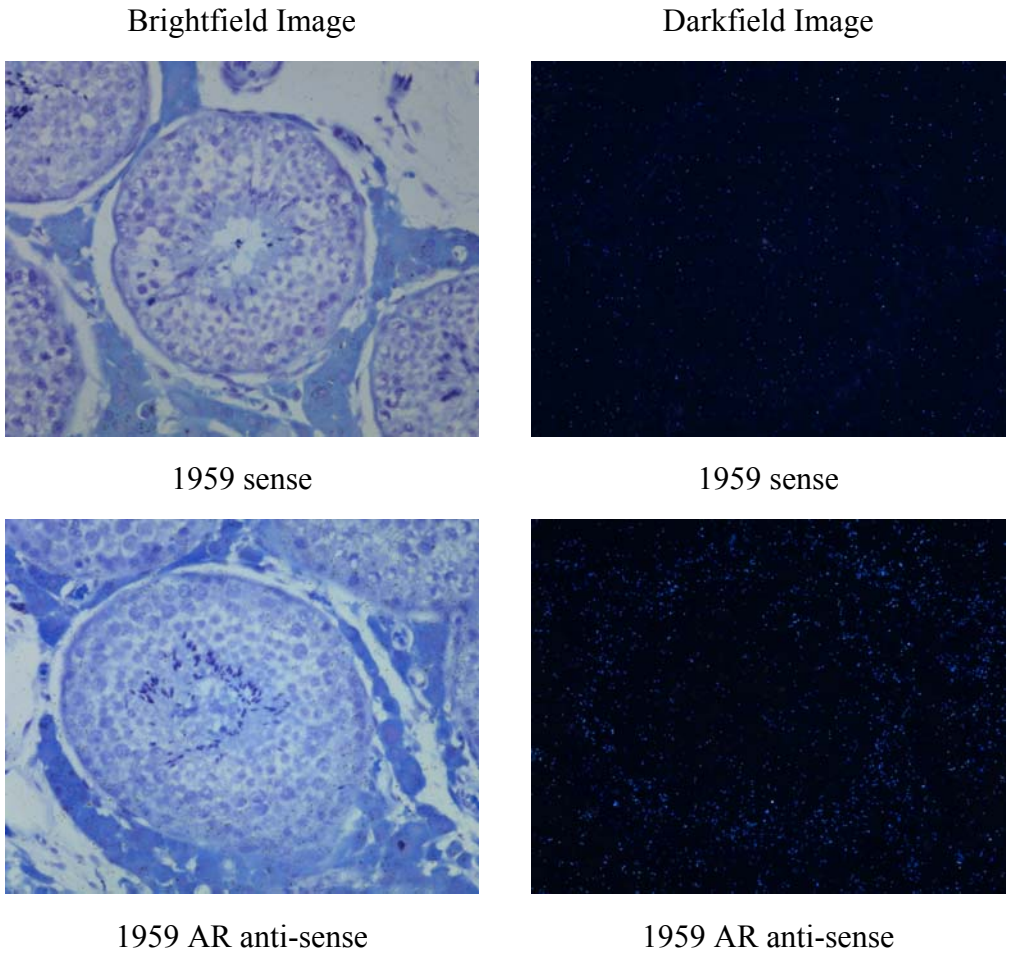


Figure 17. *In situ* localization results for the androgen receptor (AR) in the stallion testis. AR mRNA localized primarily to the interstitial space and the perimeter of the seminiferous tubules, but was not present in the center of the seminiferous tubules. This would confirm the presence of AR in Leydig and Sertoli cells but not present in spermatozoa. $^{35}$S-labeled anti-sense and sense probes were hybridized to testis cross-sections in a normal stallion (animal number 1959). Brightfield and darkfield images are displayed.

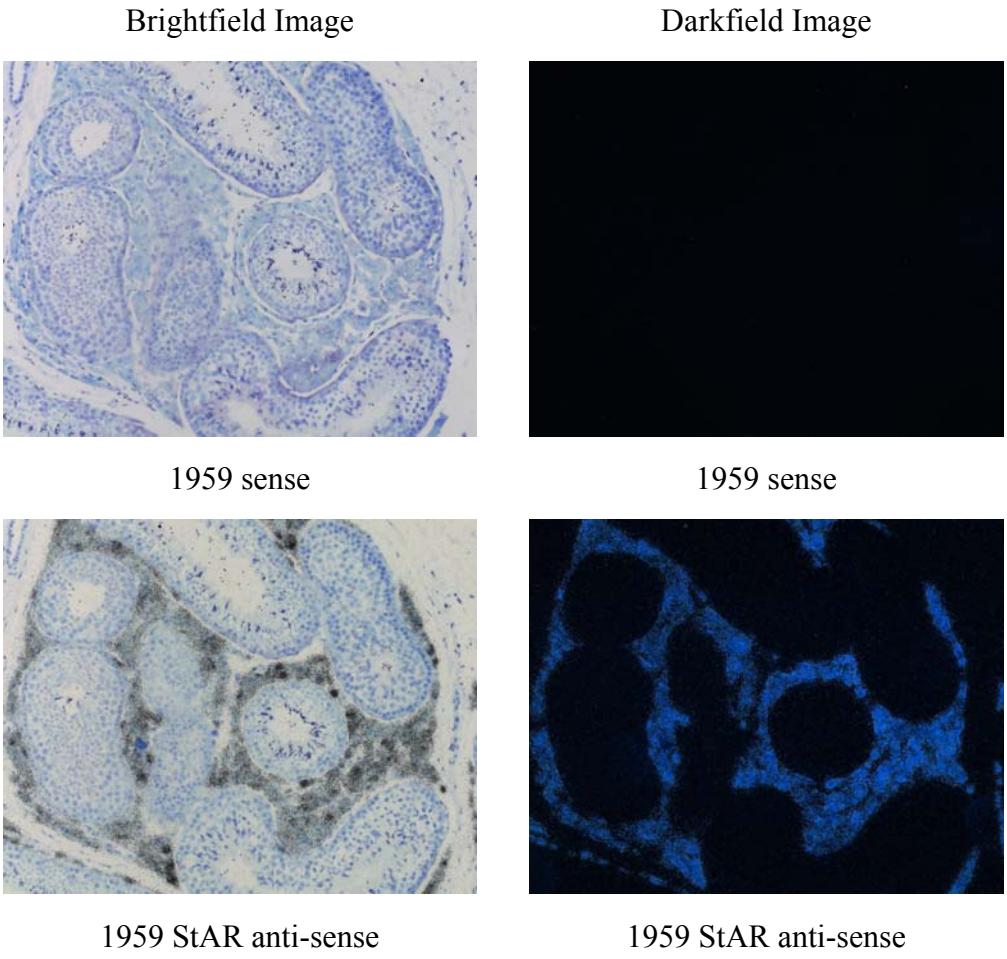


Figure 18. *In situ* localization results for steroidogenic acute regulatory (StAR) protein in the stallion testis. $^{35}$S-labeled anti-sense and sense probes were hybridized to testis cross-sections in a normal stallion (animal number 1959). Brightfield and darkfield images are displayed.

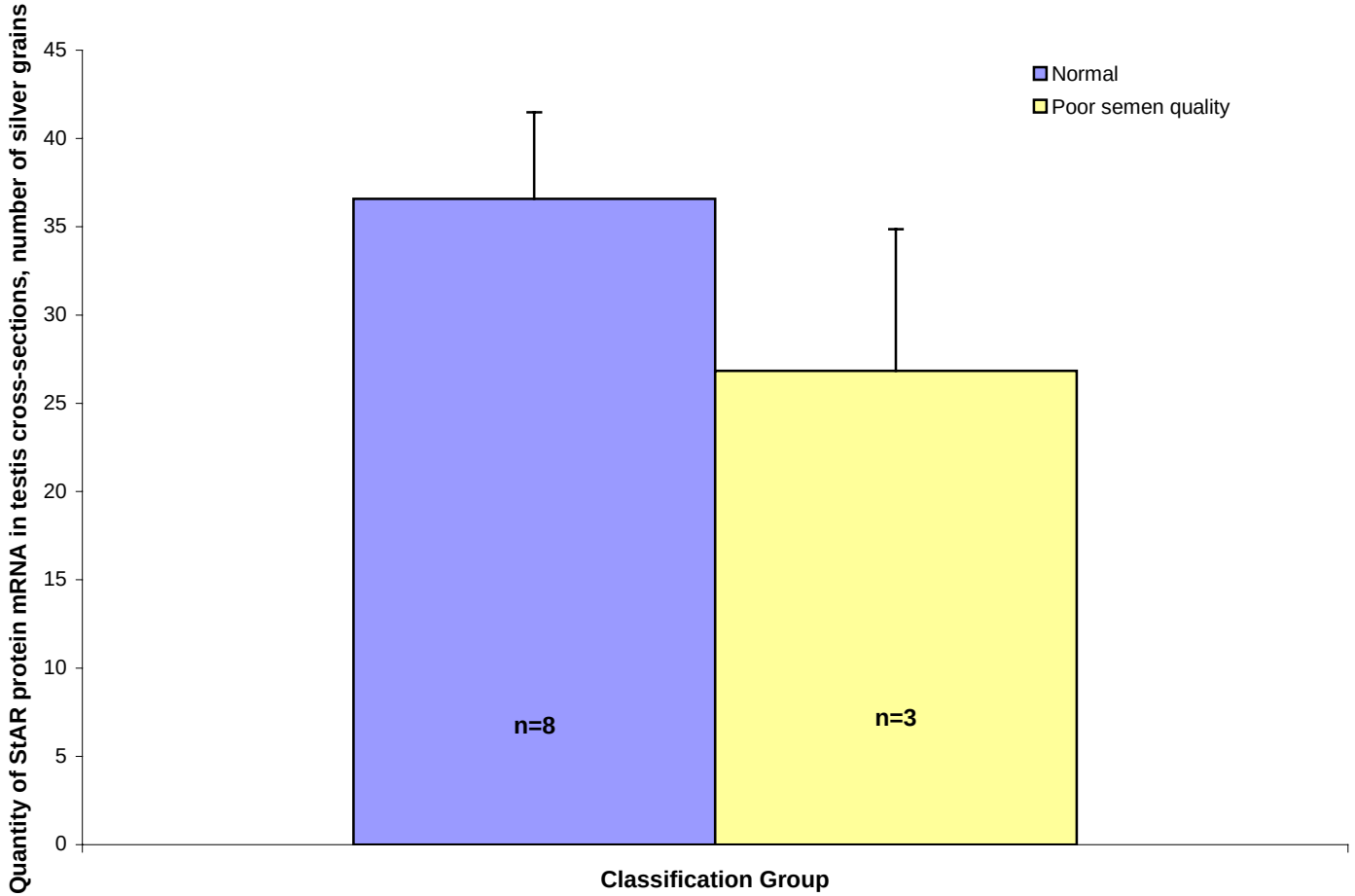


Figure 19. Quantity of steroidogenic acute regulatory (StAR) protein mRNA (number of silver grains) in Leydig cells of testis cross-sections of normal and poor semen quality stallions. Means do not differ between classification groups (P>0.05).

Synthesized probes for GR alpha, GR beta, GR exon 2 and LHR were hybridized to adrenal, pituitary, thyroid, liver, spleen and testis mRNAs. GR alpha, GR beta and GR exon 2 probes hybridized to mRNA in adrenal, pituitary, thyroid, liver, spleen and testis tissue as expected. The LHR probe only hybridized to testis mRNA as expected.

The quantity of specific mRNAs in the testis sample of each stallion was measured using slot blot hybridization techniques (Figure 20). The quantity of FSHR, LHR, StAR, GR alpha, GR exon 2, AR, IGF-I and IGF-II mRNAs (Figures 21 to 28) did not differ between normal and poor semen quality stallions. The quantity of ER alpha and GR beta mRNAs (Figures 29 and 30) had a tendency to be different ($P<0.10$) between normal and poor semen quality stallions. The quantity of ER beta, βB inhibin, PRLR, GHR and IGF-IR mRNAs (Figures 31 to 35) were significantly different ($P<0.05$) between normal and poor semen quality stallions. Numerical results of slot blot mRNA data are reported in Table 3.

**Discussion**

To date, the StAR protein has been detected in Leydig cells in the human, mouse, rat, bull and pig (Pollack et al., 1997; Aspden et al., 1998; Thompson et al., 1999; Garmey et al., 2000; Kotula et al., 2001). Similarly, in this study StAR protein mRNA localized to Leydig cells in the interstitial space of the stallion testis. There appears to be no difference in the amount of StAR protein mRNA present in the testis of normal and poor semen quality stallions used in this study. The presence of StAR protein mRNA in the Leydig cells is important for cholesterol transport from the outer mitochondrial membrane to the inner mitochondrial membrane for steroid biosynthesis (Pollack et al., 1997).

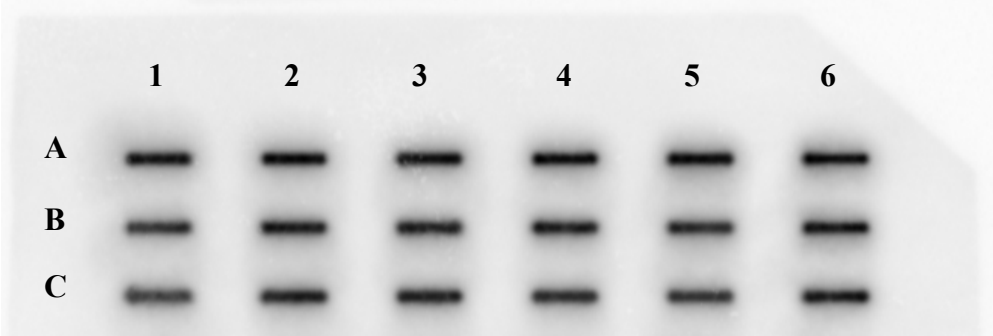


Figure 20. Representative slot blot of IGF-II mRNA in tissue from stallion testis samples. Wells A1 through A6 represent one row containing six different RNA samples. The slot blot apparatus is able to hold 48 different RNA samples at one time for RNA analysis. Stallion testis sample locations are as follows: A1=1859, A2=Lucky left testis, A3=1906, A4=1861, A5=Peppy, A6=1959, B1= 1957, B2=Peanut Butter left testis, B3=Lucky right testis, B4=7-5, B5=King's Travelin Dude, B6=1955, C1=Arab, C2=Leo Castelli, C3=Peanut Butter right testis, C4=Millenium Impulse, C5=State Dancer right testis and C6=State Dancer left testis. Samples were taken from the left testis unless stated otherwise. Not all of the stallions listed above were used in this study. The slot blot image was obtained using the Typhoon 8600 scanner variable mode imager.

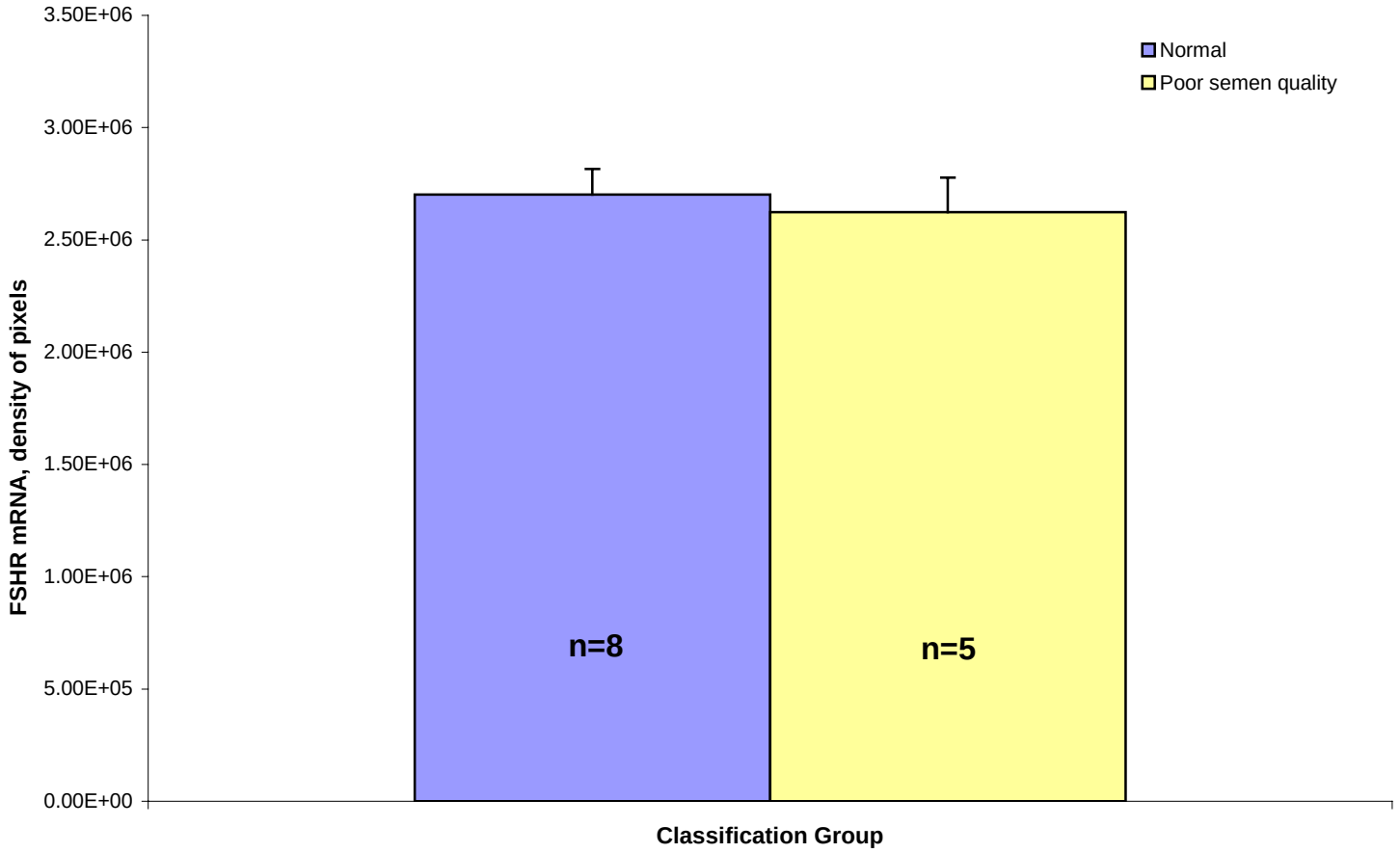


Figure 21. Quantity of follicle-stimulating hormone receptor (FSHR) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means do not differ between classification groups (P>0.05).

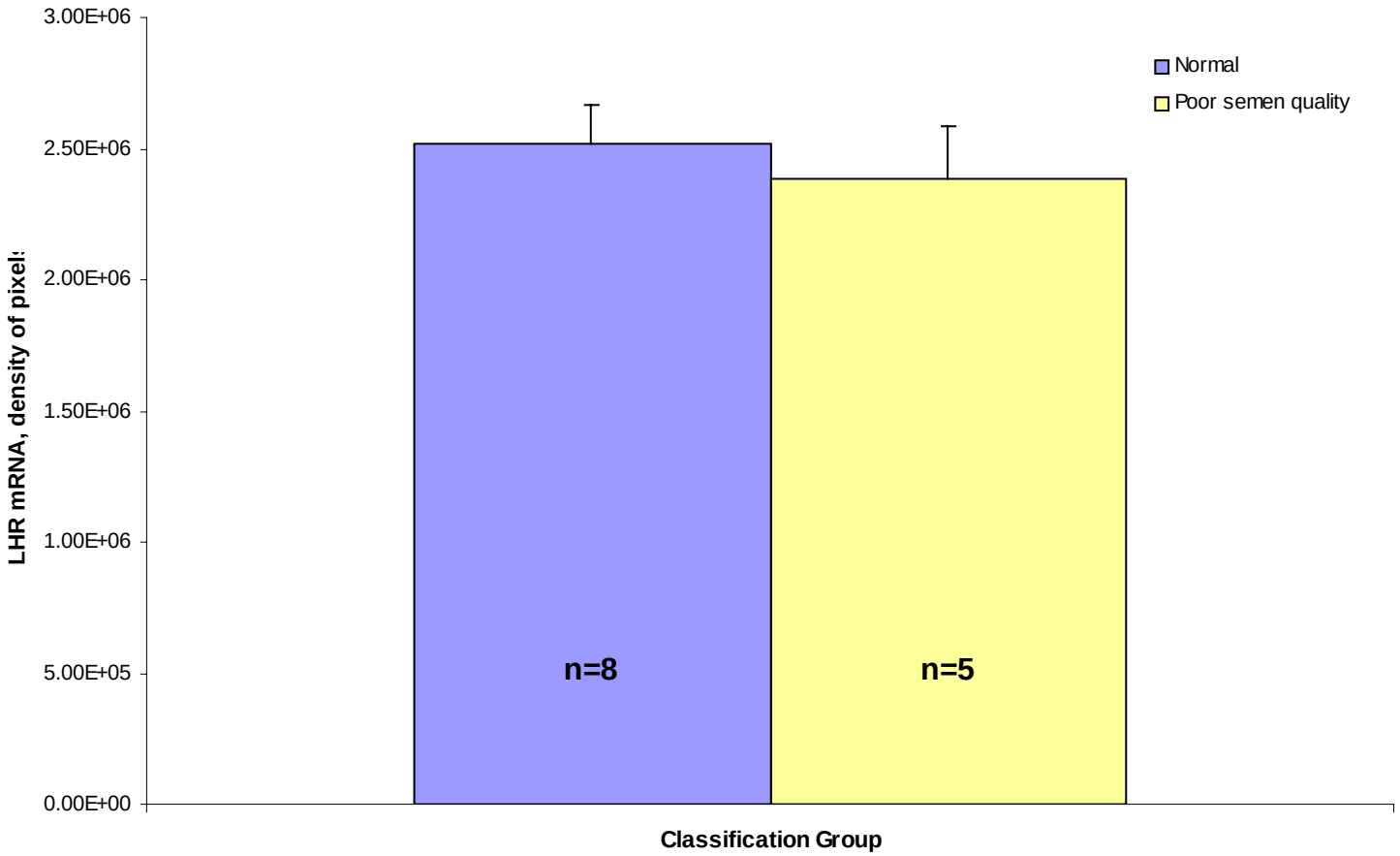


Figure 22. Quantity of luteinizing hormone receptor (LHR) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means do not differ between classification groups (P>0.05).

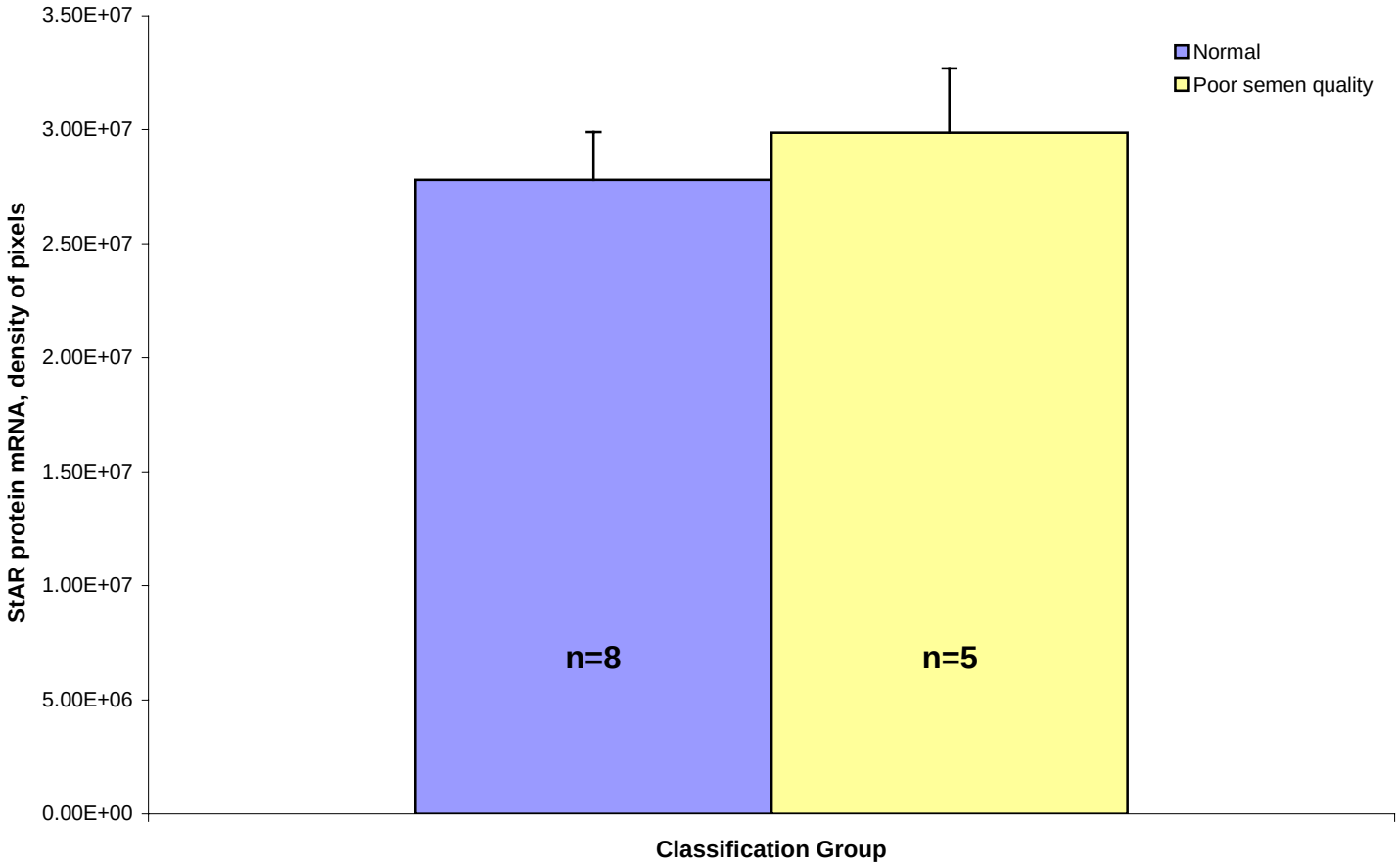


Figure 23. Quantity of steroidogenic acute regulatory (StAR) protein mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means do not differ between classification groups (P>0.05).

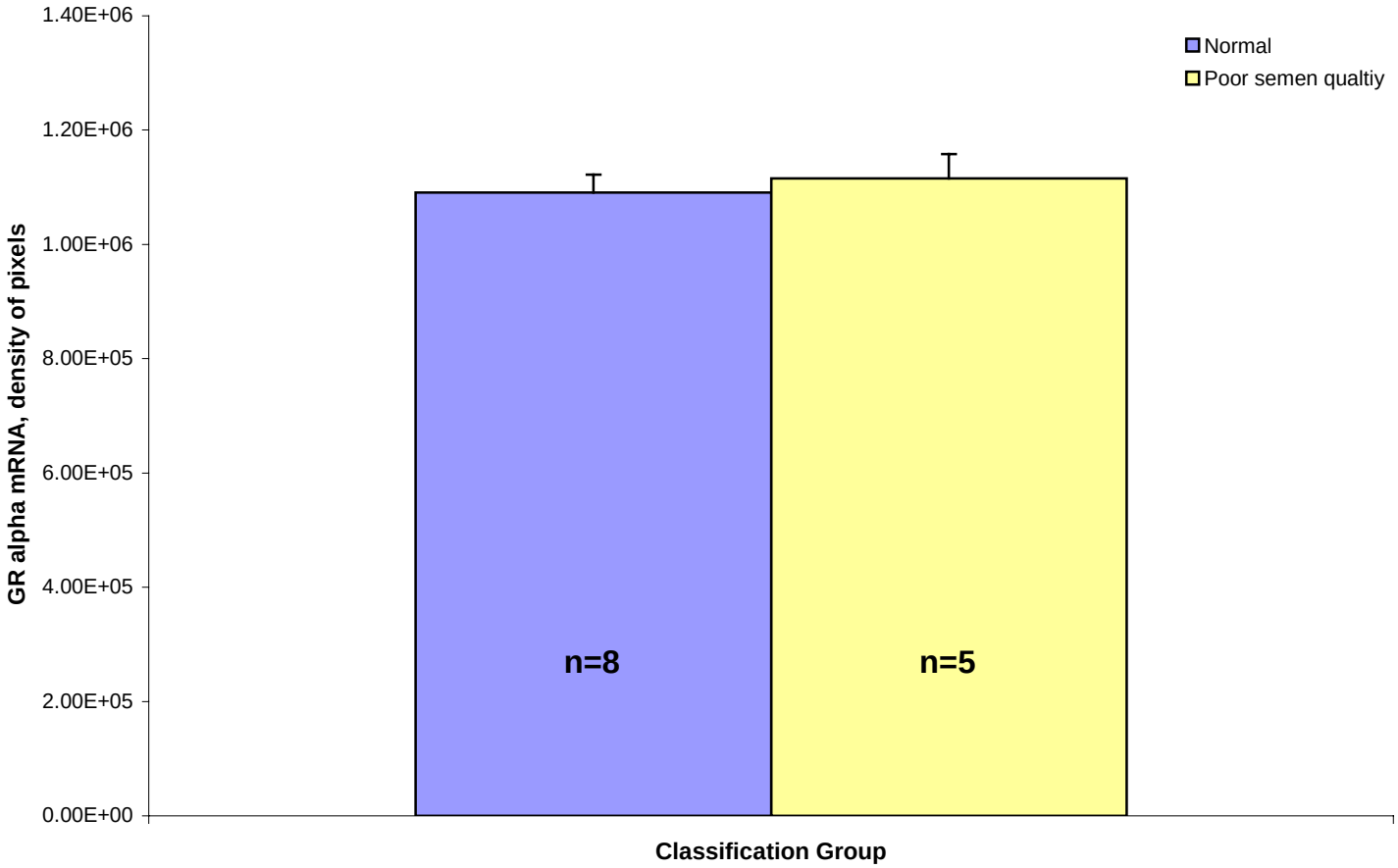


Figure 24. Quantity of glucocorticoid receptor alpha (GR alpha) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means do not differ between classification groups (P>0.05).

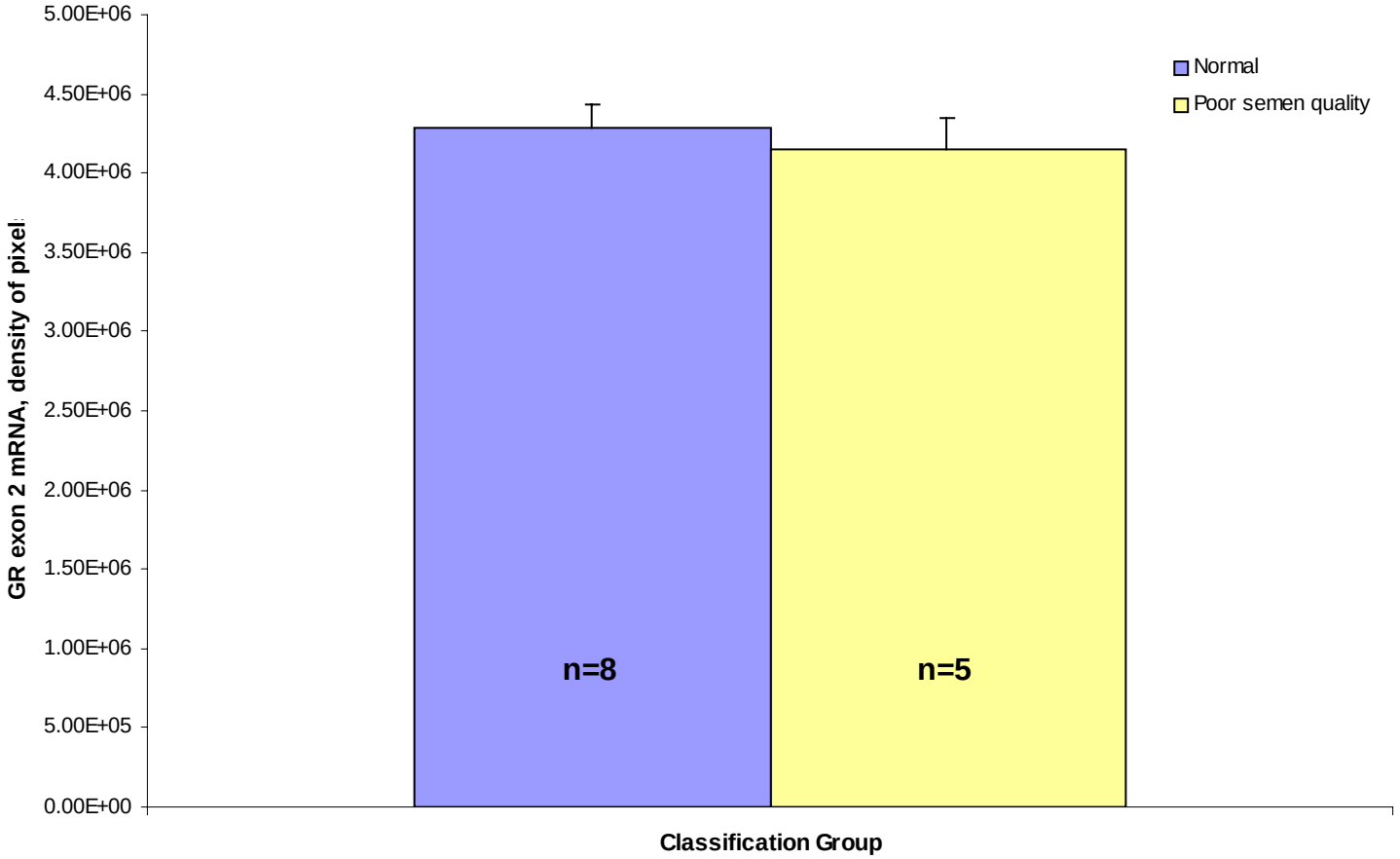


Figure 25. Quantity of glucocorticoid receptor exon 2 (GR exon 2) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means do not differ between classification groups (P>0.05).

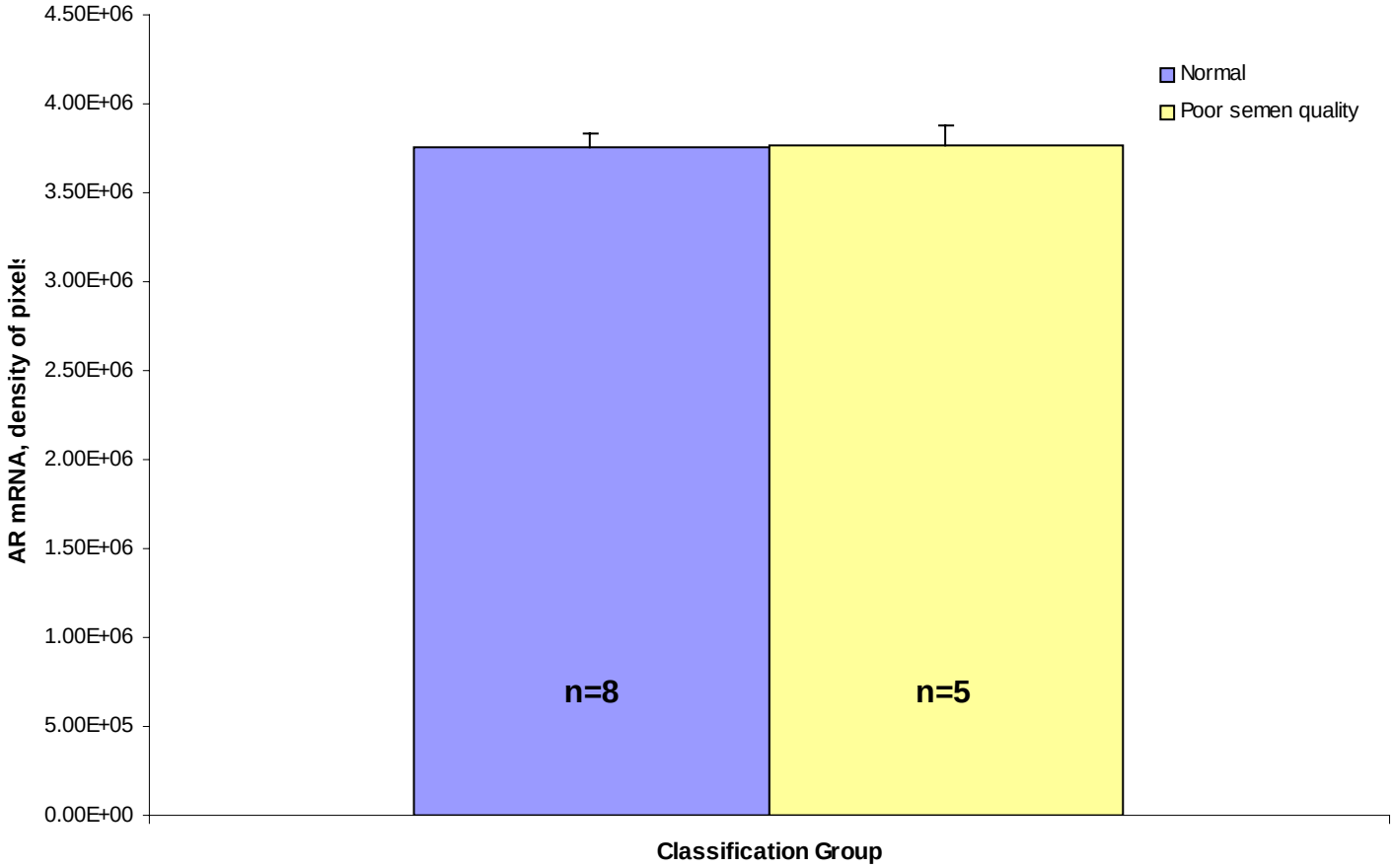


Figure 26. Quantity of androgen receptor (AR) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means do not differ between classification groups (P>0.05).

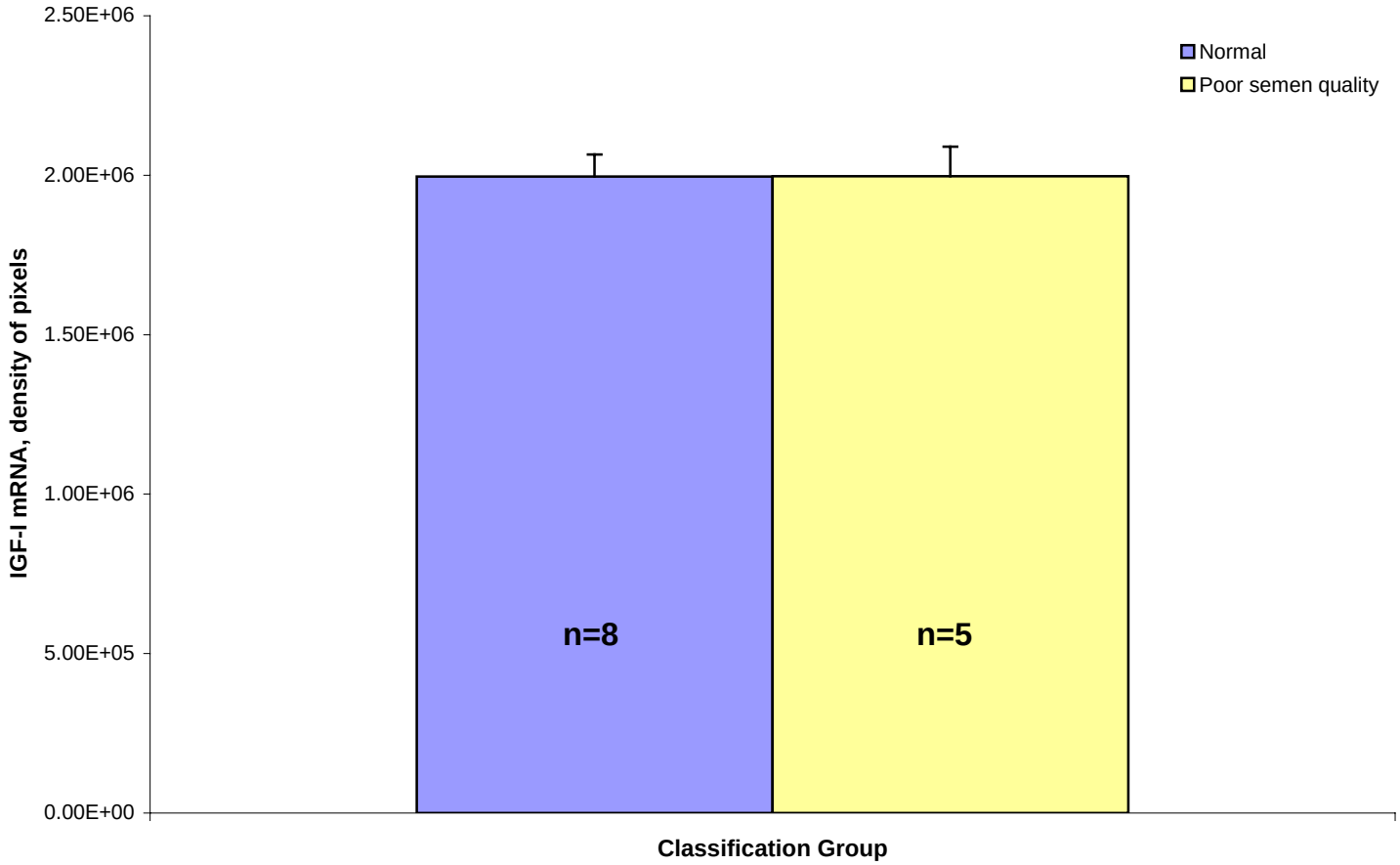


Figure 27. Quantity of insulin-like growth factor I (IGF-I) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means do not differ between classification groups (P>0.05).

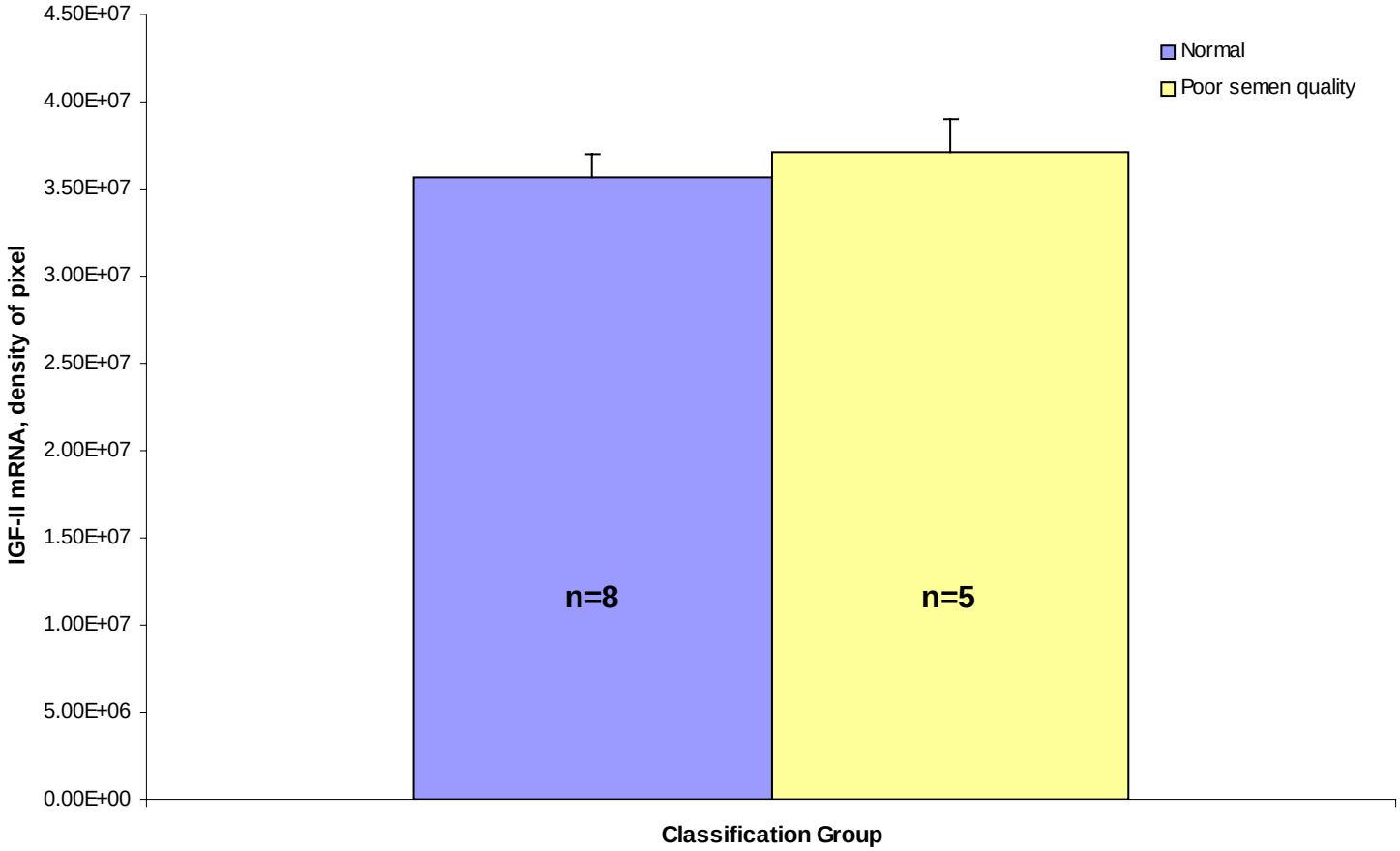


Figure 28. Quantity of insulin-like growth factor II (IGF-II) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means do not differ between classification groups (P>0.05).

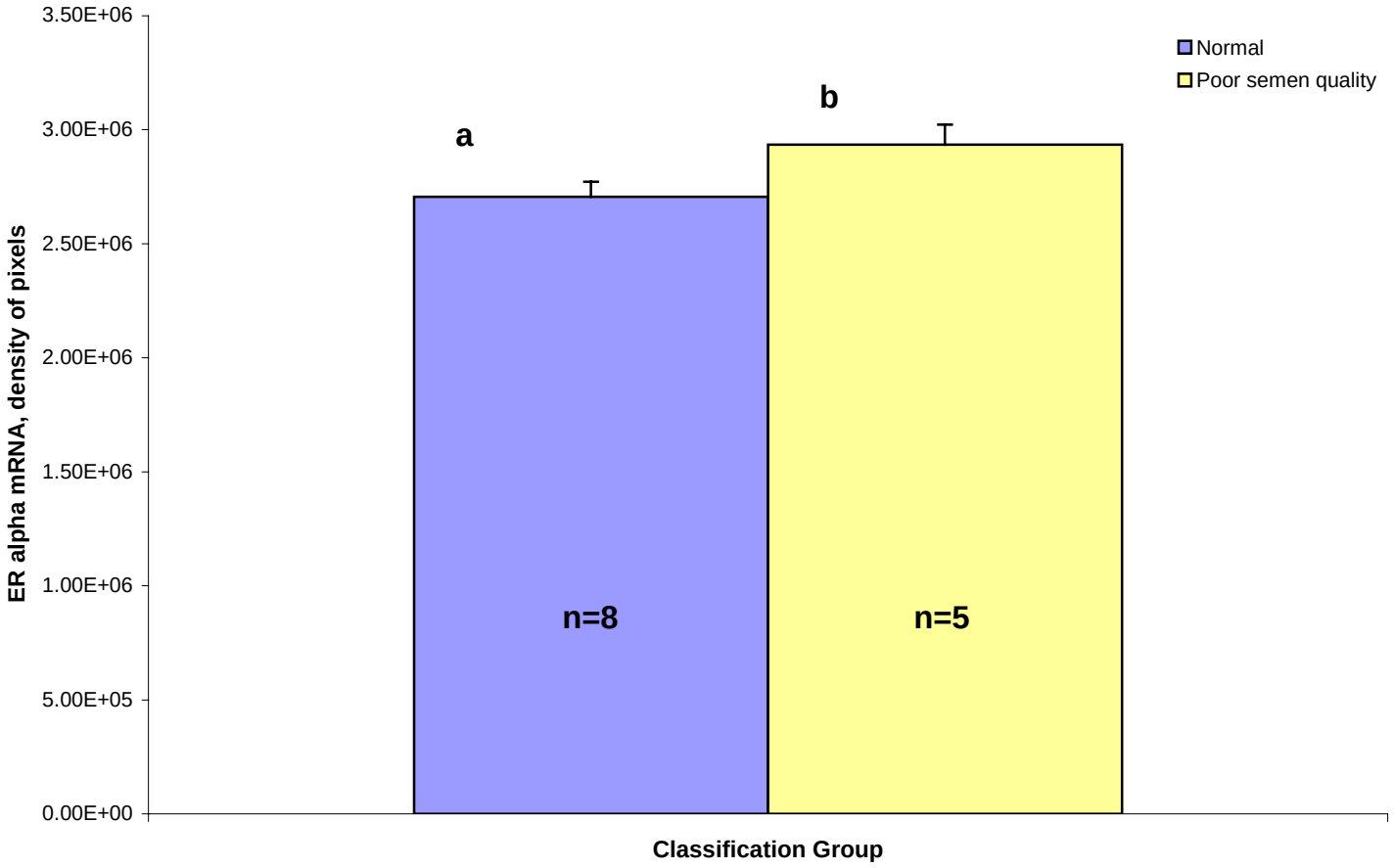


Figure 29. Quantity of estrogen receptor alpha (ER alpha) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means with different subscripts differ (P<0.10).

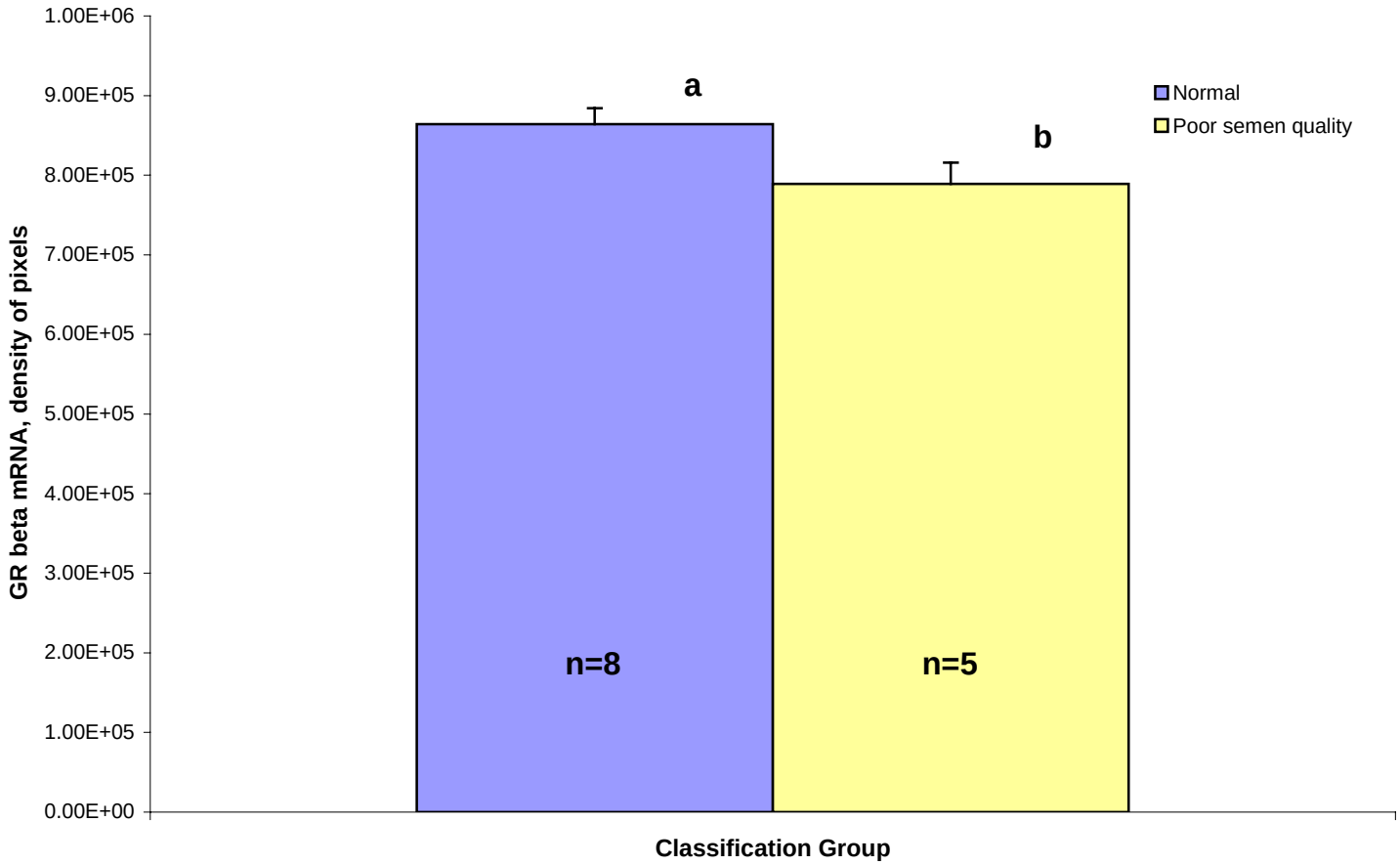


Figure 30. Quantity of glucocorticoid receptor beta (GR beta) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means with different subscripts differ (P<0.10).

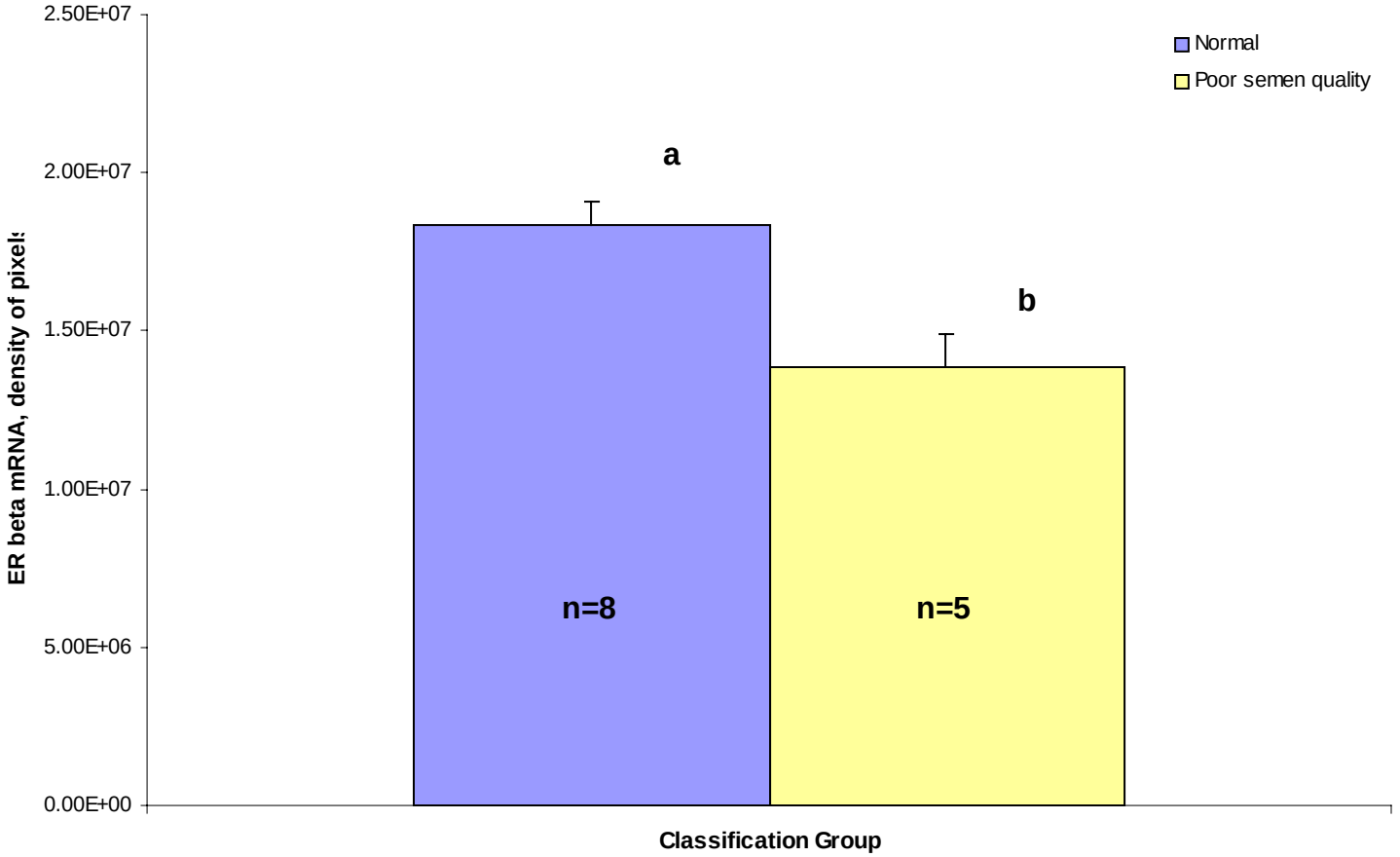


Figure 31. Quantity of estrogen receptor beta (ER beta) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means with different subscripts differ ($P<0.05$).

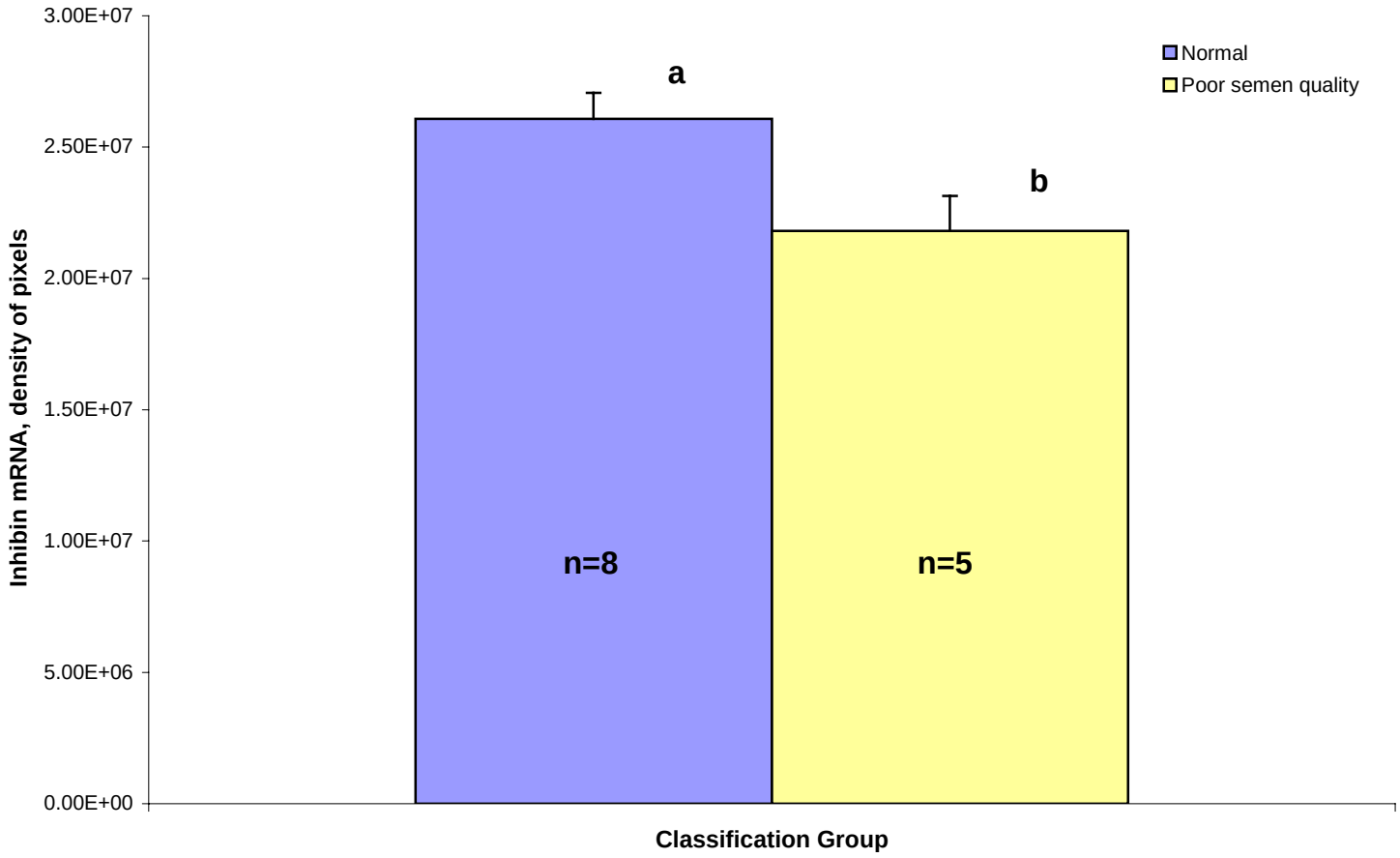


Figure 32. Quantity of βB inhibin mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means with different subscripts differ (P<0.05).

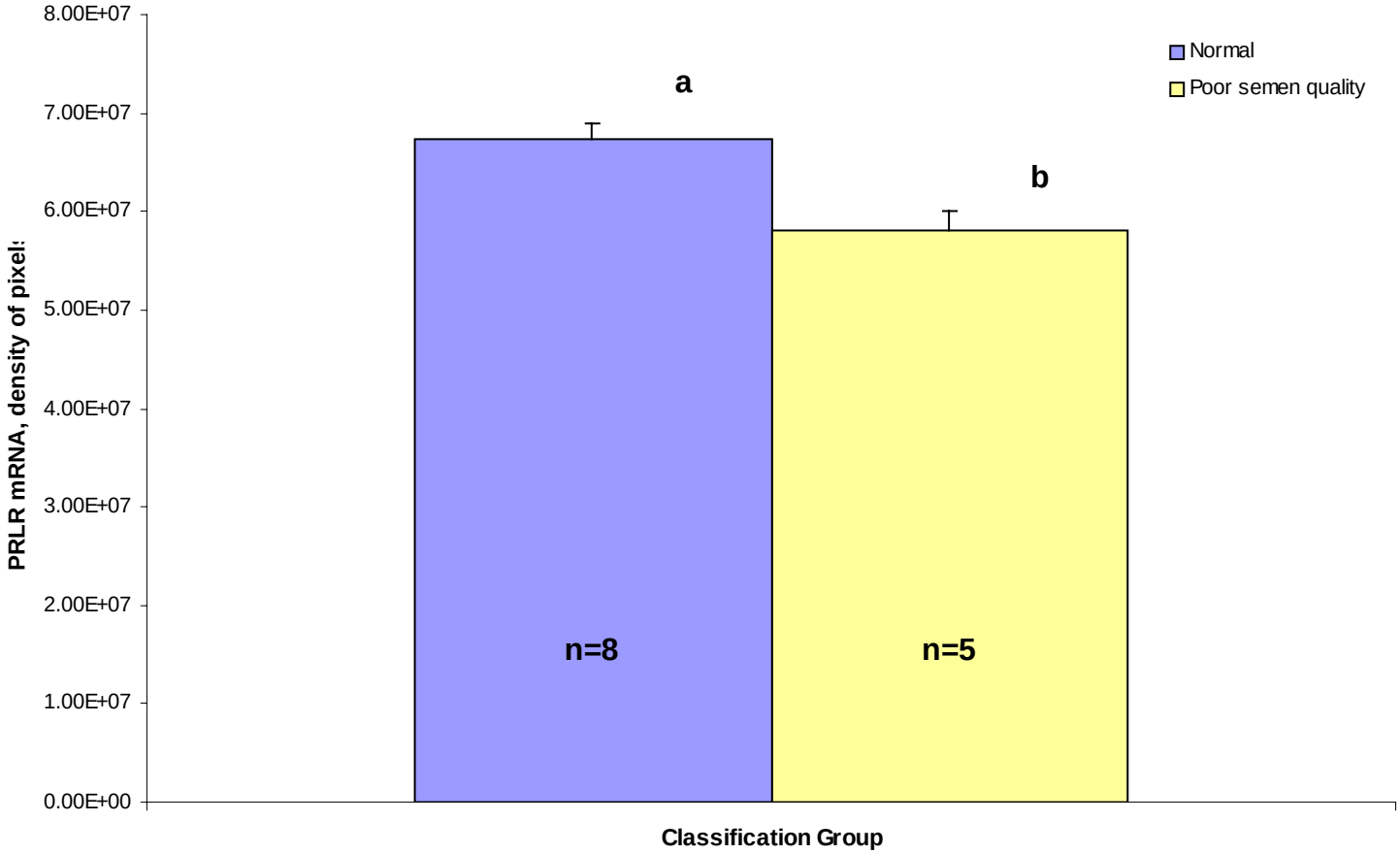


Figure 33. Quantity of prolactin receptor (PRLR) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means with different subscripts differ ($P<0.05$).

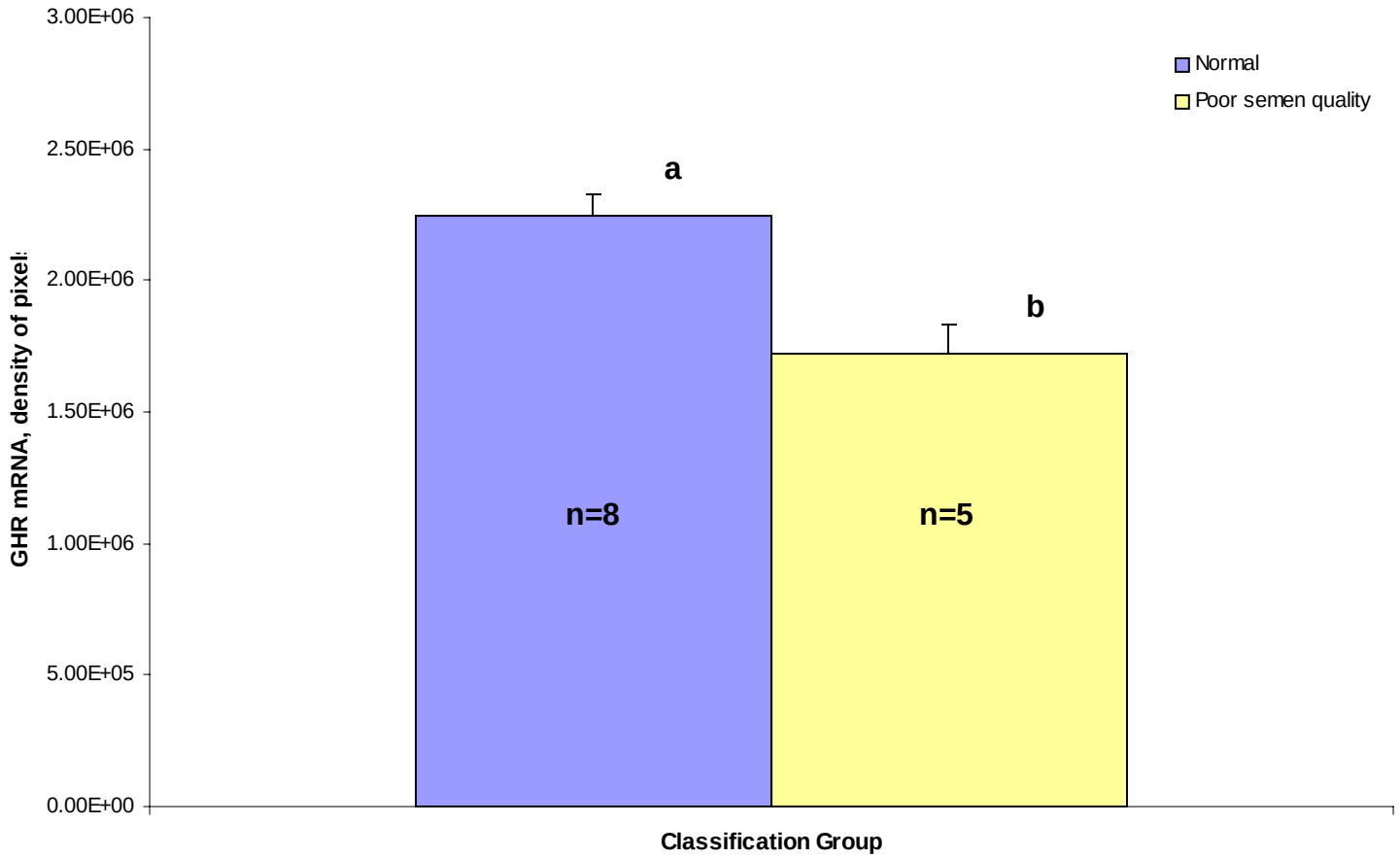


Figure 34. Quantity of growth hormone receptor (GHR) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means with different subscripts differ ($P<0.05$).

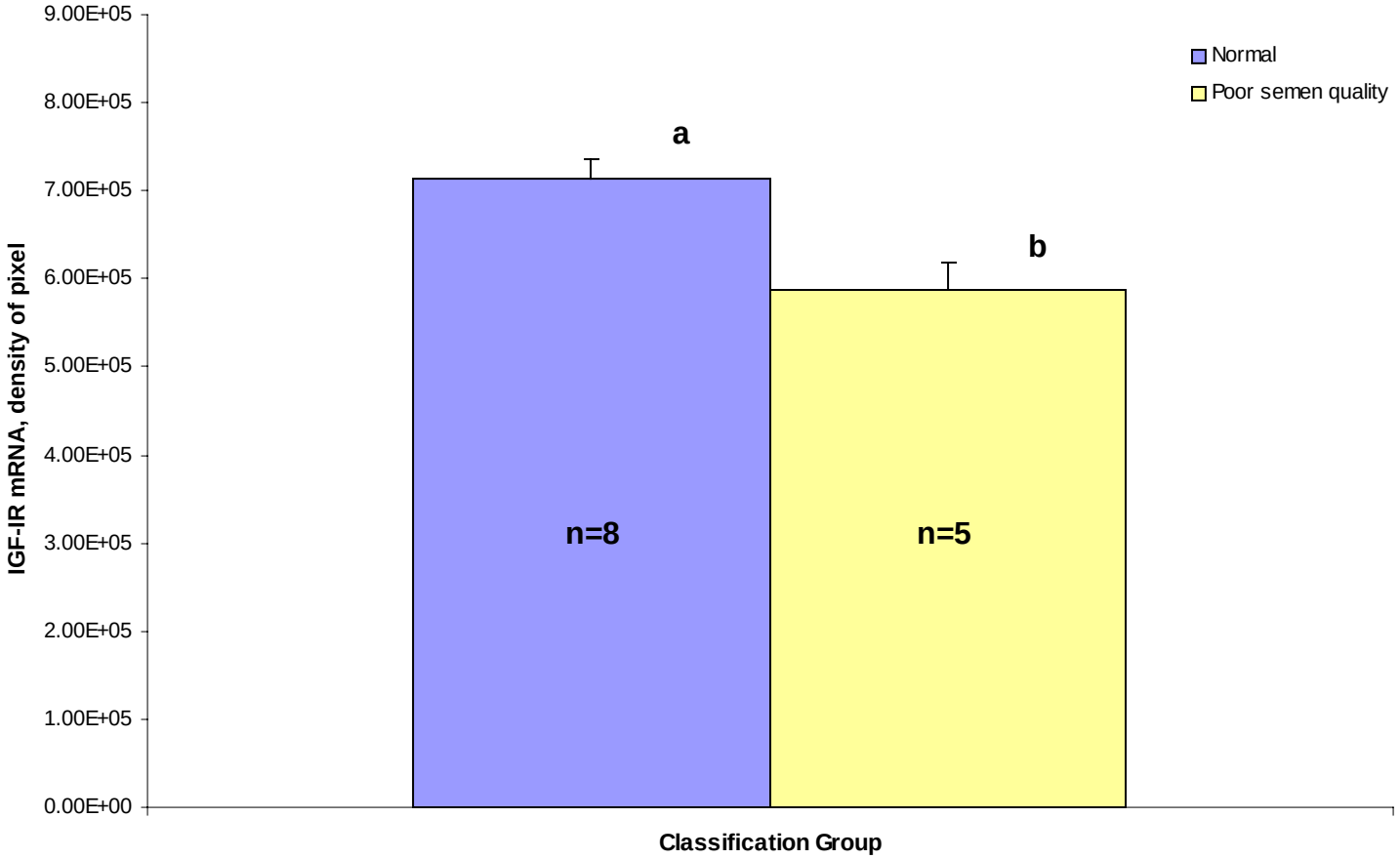


Figure 35. Quantity of insulin-like growth factor I receptor (IGF-IR) mRNA (density of pixels) in the testis of normal and poor semen quality stallions. Means with different subscripts differ (P<0.05).

Table 3. Summation of slot blot mRNA results (density of pixels) for FSHR, LHR, StAR, GR alpha, GR exon 2, AR, IGF-I, IGF-II, ER alpha, GR beta, ER beta, βB inhibin, PRLR, GHR and IGF-IR mRNAs in normal and poor semen quality stallion testis RNA. Comparison is between normal and poor semen quality stallion testis samples within gene and not between gene.

| mRNA | Normal Stallion Mean (density of pixels) | Normal Stallion Standard Deviation (density of pixels) | Poor Semen Quality Stallion Mean (density of pixels) | Poor Semen Quality Stallion Standard Deviation (density of pixels) | P-value |
|---|---|---|---|---|---|
| **FSHR** | 2.70x10e6 | 1.15x10e5 | 2.62x10e6 | 1.55x10e5 | 0.72 |
| **LHR** | 2.52x10e6 | 1.49x10e5 | 2.38x10e6 | 2.01x10e5 | 0.64 |
| **StAR** | 2.78x10e7 | 2.09x10e6 | 2.99x10e7 | 2.82x10e6 | 0.61 |
| **GR alpha** | 1.09x10e6 | 3.12x10e4 | 1.12x10e6 | 4.21x10e4 | 0.69 |
| **GR exon 2** | 4.29x10e6 | 1.48x10e5 | 4.15x10e6 | 2.00x10e5 | 0.63 |
| **AR** | 3.75x10e6 | 7.88x10e4 | 3.77x10e6 | 1.06x10e5 | 0.91 |
| **IGF-I** | 2.00x10e6 | 6.87x10e4 | 2.00x10e6 | 9.27x10e4 | 0.99 |
| **IGF-II** | 3.56x10e7 | 1.40x10e6 | 3.71x10e7 | 1.90x10e6 | 0.58 |
| **ER alpha** | 2.71x10e6 | 6.54x10e4 | 2.93x10e6 | 8.83x10e4 | 0.09 |
| **GR beta** | 8.64x10e5 | 1.99x10e4 | 7.89x10e5 | 2.69x10e4 | 0.07 |
| **ER beta** | 1.84x10e7 | 7.53x10e5 | 1.39x10e7 | 1.02x10e6 | 0.01 |
| **βB inhibin** | 2.61x10e7 | 9.86x10e5 | 2.18x10e7 | 1.33x10e6 | 0.04 |
| **PRLR** | 6.73x10e7 | 1.57x10e6 | 5.80x10e7 | 2.12x10e6 | 0.01 |
| **GHR** | 2.24x10e6 | 8.11x10e4 | 1.72x10e6 | 1.09x10e5 | 0.01 |
| **IGF-IR** | 7.14x10e5 | 2.27x10e4 | 5.87x10e5 | 3.06x10e4 | 0.01 |

GR is another important endocrine gene in the stallion. In this study, GR exon 2 mRNA localized in a general pattern over the interstitial space and within the seminiferous tubule to cell types throughout the testes. Previously, GR was identified in Leydig cells, Sertoli cells and germ cells in the testes of the human and rat (Weber et al., 2000). AR mRNA localized primarily to the interstitial space and the perimeter of the seminiferous tubules, but was not present in the center of the seminiferous tubules. This would confirm the presence of AR in Leydig and Sertoli cells but not present in spermatozoa. Previously, the presence of AR was reported in Leydig and Sertoli cells in the human, mouse, rat and pig testes (Namiki et al., 1991; Ruizeveld de Winter et al., 1991; Kotula et al., 2000; Pelletier et al., 2000; Zhou et al., 2002).

In the future, the slot blot northern blot technique should be tested further to determine the optimal amount of RNA that binds the nylon membrane. This will determine if the 18S ribosomal RNA control binds in excess on the membrane, which is necessary for accurate results. This is a potential problem with the data reported in this study and results would need to be confirmed with lower amounts of RNA bound to the membrane if the 18S ribosomal RNA control is not in excess with 20 μg of RNA per slot.

LHR mRNA is present in stallion testes, but not in adrenal gland, pituitary gland, thyroid liver or spleen and has been shown in other species. This confirms the specificity of the newly designed LHR probe. GR alpha, GR beta and GR exon 2 mRNAs are present in various stallion tissues besides the testes, including the adrenal gland, pituitary gland, thyroid, liver and spleen. These results are the same as observed by other investigators in the human and rat (Weber et al., 2000; Pujols et al., 2002). Both isoforms GR alpha and beta were present in stallion testes. Previously this information

was unknown since the synteny of GR beta is not conserved across species and is absent in the mouse (Otto et al., 1997). GR beta is hypothesized to be involved in blocking ligand binding to GR alpha, thereby causing glucocorticoid resistance in the animal. Because of this, the use of long-term glucocorticoid treatment in the horse should be reconsidered to avoid problems with glucocorticoid resistance. Further studies are necessary to determine the physiological significance of GR beta in the stallion testis and the effects of glucocorticoids on reproduction.

The quantity of FSHR and LHR mRNAs were not different in testes of normal and poor semen quality stallions, indicating there is not a correlation between gonadotroph secretion or gonadotroph receptor binding and semen quality. These stallions of poor semen quality were not different in FSH concentration than stallions with normal semen quality. There was no difference in the quantity of StAR protein or AR mRNA in the testis between normal and poor semen quality stallions. Therefore, StAR protein transport of cholesterol to the inner mitochondrial membrane for steroid production and the use of androgens by binding the AR in the Leydig and Sertoli cells was not the cause of poor semen quality. Also, the quantity of GR alpha, GR exon 2, IGF-I and IGF-II mRNAs was not different between normal and poor semen quality stallions. This would indicate that glucocorticoid action in the testis was not the cause of poor semen quality. But, if the GR is present, it could be dependent on ligand and its availability for ligand to be different in poor semen quality. Also, the IGF-I and IGF-II growth factors are not responsible for poor semen quality in the stallion.

The quantity of ER alpha mRNA had a tendency ($P<0.10$) to be higher in the testes of poor semen quality stallions. This could indicate that estrogen production in the

testis is important in paracrine/autocrine regulation of spermatogenesis and normal testicular function. The role of estrogen and estrogen receptors in the testis remains unclear. Further investigations with a larger number of normal and poor semen quality stallions are needed in order to determine if there is indeed an effect of estrogen on semen quality. GR beta mRNA quantity had a tendency to be lower in the testes of poor semen quality stallions. The functional role of GR beta is still unknown. The lack of GR beta in the mouse raises questions about physiological significance of the gene isoform since the GR alpha isoform is known to be the biologically active form in humans and rats (Otto et al., 1997; Pujols et al., 2002). Further studies are needed to determine the importance and necessity of GR beta in the stallion testes and its functional role in spermatogenesis and steroidogenesis.

There were several mRNAs expressed in differing quantities in normal and poor semen quality stallions. ER beta mRNA was significantly lower in poor semen quality stallions than in normal stallions. While ER alpha knockout mice are completely infertile, ER beta knockout mice appear to have no compromised fertility (Couse et al., 2001). This would indicate that the presence of ER alpha in the stallion testis is required for fertility but the varying importance of the two isoforms remains unknown. Further studies are necessary in order to elucidate the different roles of ER alpha and ER beta in the stallion testis. The quantity of βB inhibin mRNA was lower in the poor semen quality stallion than in the normal stallion testes. Since inhibin is an important regulator of gonadotroph secretion and is important in paracrine regulation of testicular function (Marchetti et al., 2003), changes in inhibin secretion could be an indicator of fertility status (von Eckardstein et al., 1999; Hu et al., 2003). There appears to be an association

between circulating inhibin concentration and level of stallion fertility (Stewart and Roser, 1998) such that fertile stallions have higher circulating concentrations of inhibin and higher mRNA expression of inhibin in the testis. Further investigation is needed in order to understand the changes in mRNA expression in poor semen quality stallions.

Differences in mRNA expression were observed for several somatogenic/lactogenic gene receptors in the poor semen quality stallions. The quantity of PRLR, GHR and IGF-IR mRNAs were all lower in poor semen quality stallions than in normal stallions. Hormones such as prolactin, growth hormone and IGF-I appear to play an important autocrine/paracrine role in the testis (Spiteri-Grech and Nieschlag, 1993; Gnessi et al., 1997; Schlatt et al., 1997). IGF-I has been shown to stimulate proliferation of Sertoli cells and spermatogonia through IGF-IR (Jaillard et al., 1987; Soder et al., 1992). Poor semen quality stallions had a reduced GHR mRNA expression. Similarly, GHR knockout mice were shown to have reduced fertility (Chandrashekar et al., 1999). Further investigation is needed in the stallion to determine the affects of PRLR, GHR and IGF-IR expression on fertility.

This research supplies information about gene expression in the stallion testis as well as preliminary information useful in the study of semen quality. Future studies are needed in order to understand the causes of poor semen quality in the stallion.

# CHAPTER V

# SEMEN EVALUATION AND *IN VITRO* TESTIS CULTURE IN NORMAL AND POOR SEMEN QUALITY STALLIONS

## Introduction

Spermatogenesis and steroidogenesis are important functions of the testis and are necessary for fertility of an animal. Spermatogenesis is a developmental process that results in the production of sperm in the male while steroidogenesis is the pathway by which steroid hormones are produced from cholesterol (Senger, 1999). Breeding soundness exams (BSE) are used to examine the reproductive organs, the behavior, the quantity and quality of ejaculate and the function of the reproductive endocrine system (Juhasz et al., 2000). Due to the high individual value of stallions and the relative incidence of fertility disorders, there is interest in detection methods for fertility parameters (Juhasz et al., 2000). Daily sperm production (DSP) and DSP per gram of parenchyma referred to as spermatogenic efficiency are two parameters used to quantitate sperm production (Senger, 1999). Hormone measurements are also used to evaluate the function of the reproductive system of an animal. Measuring baseline concentrations of reproductive hormones occasionally identifies hormonal imbalances that could be associated with subfertility (Juhasz et al., 2000).

Normal spermatogenesis is a relatively inefficient process that results in the loss of a large number of potential spermatozoa (Clermont, 1962; Huckins, 1978). Apoptosis or programmed cell death has been implicated in the testis as an essential mechanism for removal of developing germ cells from the seminiferous epithelium (Knudson et al., 1995; Rodriguez et al., 1997; Russell et al., 2002) and has recently been demonstrated in

the testis of normal stallions (Heninger et al., 2004). Since basal levels of germ cell apoptosis have been identified for normal stallions, further research evaluating the role of apoptosis in the poor semen quality stallion is possible and would add useful information for the understanding of molecular mechanisms in the testis.

Normal spermatogenesis is dependent upon a functional hypothalamic-pituitary-testicular (HPT) axis, which involves classic endocrine actions of gonadotropins, feedback mechanisms of steroids and proteins and paracrine/autocrine modulation (Roser, 2001). This includes such hormones as LH, FSH, testosterone, estradiol, inhibin and IGF-I as well as many others. LH, through the production of testosterone, and FSH hormones are required by the testis for maintenance of spermatogenesis (De Kretser et al., 1995). Also, testicular estrogen appears to regulate reproductive function in the stallion (Thompson and Honey, 1984; Muyan et al., 1993). Numerous studies have demonstrated that testosterone and estradiol inhibit LH secretion and inhibin inhibits FSH secretion (De Kretser et al., 1995). Since all of these hormones create homeostasis in the testis for spermatogenesis, information could be gained by demonstrating hormonal changes that relate to poor semen quality. In stallions, a positive correlation exists between plasma immunoreactive (ir)-inhibin and testicular activity (Nagata et al., 1998a). Motton and Roser (1997) indicate that plasma LH, FSH, estradiol and testosterone concentrations are not different between normal and poor semen quality stallions, but that LH and FSH were significantly higher and estradiol, testosterone and inhibin concentrations were significantly lower in infertile stallions. Also, Hess and Roser (2001) state that plasma IGF-I concentration does not have a direct relationship with declining fertility in stallions.

*In vitro* culture methods are useful to test specific questions about tissue function in a more controlled environment. Culturing testicular parenchyma allows for addition of compounds such as hCG, which elicits an LH-like response without affecting FSH activity, and forskolin. HCG stimulation tests the LHR signaling mechanisms in the stallion testis, while forskolin stimulates the second messenger system through activation of adenylate cyclase bypassing the LH receptor. Both hCG and forskolin activate steroidogenesis in Leydig cells to stimulate synthesis of testosterone. Testosterone assays are then used to detect changes due to *in vitro* challenges of the testis. This is a diagnostic tool to directly test the secretory capacity of the Leydig cells (Juhasz et al., 2000).

Other changes such as gene expression in the testis can be measured to determine relationships of hormone pathways and second messenger systems. The biosynthesis of all steroid hormones begins with the transport of cholesterol from the outer mitochondria to the inner mitochondrial membrane by StAR protein (Stocco and Clark, 1996). Subsequently, the conversion of cholesterol to pregnenolone by the cytochrome P450scc enzyme complex takes place and starts the cascade of enzymatic reactions for testosterone production (Kerban et al., 1999). Previous studies have demonstrated regulation of StAR mRNA with hCG treatment (Kerban et al., 1999) which suggests that hCG challenges affect gene expression in the testis. Changes in StAR and P450scc proteins can be measured using western blot techniques to analyze differences in expression due to hCG or forskolin treatment in the testis.

By using methods such as seminal parameter measurements, apoptotic rate, hormone concentrations and *in vitro* culture techniques testicular function of an animal

can be evaluated. Further research is needed in order to define the normal limits of these parameters for stallions so that poor semen quality or infertile animals can be assessed and treated.

**Materials and Methods**

*Animals*

Testes were surgically removed under general anesthesia from eight mature (ages 4-17 years), light-breed stallions with normal testes size and semen quality and from five mature, light-breed stallions with poor semen quality. See Table 2 (page 42) for stallion identification information and semen quality descriptions. Normal and poor semen quality classification groups were sorted by daily sperm output. Slices of testicular parenchyma were harvested from the center of the testis adjacent to the central vein. Parenchymal samples were 1) snap frozen in liquid nitrogen for RNA and protein evaluation, 2) fixed in 4% paraformaldehyde for detection of apoptosis and 3) placed on ice for *in vitro* culture use.

*Semen Quality Evaluation*

**Daily Sperm Production (DSP).** Estimation of DSP was performed according to previously established methods (Amann, 1962). Briefly, 0.2 to 0.5 grams of 2% glutaraldehyde fixed testicular parenchyma were homogenized in a Waring blender for 6 minutes in 100 ml of fluid containing 150 mM NaCl, 0.05% (v/v) Triton X-100 and 3.8 mM $NaN_3$. Maturation-phase elongated spermatids were enumerated using duplicate evaluations for a single observer. DSP per gram parenchyma (DSP/g) was calculated for each horse as the number of elongated homogenization-resistant spermatids divided by

the product of the weight of the homogenized tissue and the lifespan of the spermatids (8.2 days).

**Seminal Parameters.** Daily Sperm Output (DSO) was estimated by evaluation of sperm numbers in ejaculates after extragonadal sperm reserves were stabilized (Varner et al., 1991). Sperm concentrations in an ejaculate were determined using a densimeter (Animal Reproduction Systems, Chino, CA). Semen samples were also evaluated for motility (% total motile sperm), progressive motility (% progressively motile sperm, PMS) and morphology (% morphologically normal sperm, MNS).

**Apoptosis.** Apoptosis was measured using fixed parenchymal samples as described in Heninger et al., 2004 for TUNEL immunohistochemistry. Briefly, to quantify germ cell apoptotic rates in reproductively normal and poor semen quality stallions, an ApopTag® peroxidase detection kit S7100 (TUNEL) (Intergenco, Purchase, NY, USA) was used according to manufacturer's instructions. Apoptosis quantification was performed by counting the number of TUNEL-positive germ cells per one hundred Sertoli cells to obtain a value of apoptosis for each animal.

*Hormone Assays*

Blood samples were collected via jugular venipuncture. Plasma samples were isolated by centrifugation for 10 minutes at 10,000xg. The blood samples were frozen at –20ºC. Samples were analyzed for testosterone, estradiol, luteinizing hormone (LH), follicle stimulating hormone (FSH) and inhibin by radioimmunoassay (RIA) (Courtesy of J. F. Roser, Department of Animal Science, University of California, Davis, CA). LH, FSH and testosterone RIAs were performed according to Roser and Hughes (1991). The estradiol RIA was performed following procedures from Pinaud et al. (1991) and

immunoreactive inhibin was assayed according to Roser et al. (1994). All blood samples were measured in one assay for each hormone to avoid assay variation.

*IGF-I Concentration*

The concentration of IGF-I was also measured in the blood samples of normal and poor semen quality stallions by RIA (see Appendix I for protocol) as described by Bilby et al, 1999 with a few modifications. All blood samples were measured in one assay to avoid assay variation. Briefly, 10 µl of serum was pipetted into polypropylene 12X75 tubes and extracted with 400 µl 1M glycine (pH 3.2) and 500 µl IGF-I assay buffer (pH 3.5) at 37ºC for 48 hours. Next, 90 µl of 0.5N NaOH was added to each extracted sample and vortexed to neutralize the pH to 7.0. The assay standards and acidified unknowns were then pipetted in duplicate into polypropylene 12X75 tubes using 50 µl of extracted sample volume. 100 µl of rabbit anti-human IGF-I (UB3-189; A. F. Parlow, National Hormone and Peptide Program, Torrance, CA) antibody (1:120,000 dilution) was added to assay tubes, mixed and incubated at 4ºC for 24 hours. 100 µl of $^{125}$I-IGF-I tracer (cat# 68128; MP Biomedicals (formerly ICN), Irvine, CA) was added to all assay tubes, mixed and incubated for 16 hours at 4ºC. Next, 50 µl of normal rabbit serum (NRS), 50 µl of goat anti-rabbit IgG (GARGG; 1:60 dilution) secondary antibody (Calbiochem, San Diego, CA) and 300 µl of polyethylene glycol were added to assay tubes, mixed and incubated at room temperature for 30 minutes. Assay tubes were centrifuged at 3000 rcf for 30 minutes at 4ºC and supernatant decanted into a radioactive waste container. Assay tubes were counted with a Beckman gamma counter for 1 minute. Unknown samples were analyzed using AssayZap (Biosoft Inc., Cambridge, UK) to calculate concentrations of unknowns in comparison to a known standard curve.

In Vitro *Culture*

Parenchymal samples were collected from testes of normal and poor semen quality stallions. Fresh tissue was kept on ice and transported to the laboratory for *in vitro* culture (see Appendix F for protocol). One gram of testicular parenchyma was weighed and incubated in 5 mL of DMEM (cat# 1600034; Gibco Invitrogen Corp., Carlsbad, CA) media containing Penicillin/Streptomycin (cat# 5070063; Gibco Invitrogen Corp., Carlsbad, CA) in a 50 mL tube. Samples were incubated at 34°C with 95% $O_2$/5% $CO_2$ exposure in a shaking water bath. After two hours of incubation, media was removed and frozen. Treatments either of media alone, 10 international units per ml (IU/ml) human chorionic gonadotropin (hCG; Pregnyl from Organon, West Orange, NJ), 100 IU/ml hCG or $10^{-5}$ M forskolin (cat# 80058-086; VWR, West Chester, PA) were added to the testis samples and incubated for another two hours. After two hours, the samples were divided into either 2-hr or 4-hr treatment groups. Media from the 2-hr treatment group was collected and frozen at –20°C. The testis samples were frozen in an Rnase-free tube at –80°C. The 4-hr incubation samples had 200 µl of media removed and frozen. The remainder of the media continued to incubate for another two hours. After 4-hr of incubation, the 4-hr sample media and testis tissue were collected and frozen as described above.

*Testosterone Concentrations*

Testosterone concentrations were measured using radioimmunoassay (RIA; see Appendix H for protocol) for circulating testosterone production as well as testosterone produced *in vitro* from cultured testicular cells (mentioned above) (Godfrey et al., 1990; French and Welsh, 1990). All blood samples were measured in one assay to avoid assay

variation. Blood serum or media from culture was pipetted into 12X75 polypropylene tubes and brought to 500 µl volume with PBSG diluent. 100 µl of testosterone antibody (Colorado State University) and 100 µl of $^{3}$H-testosterone tracer (Perkin Elmer-NEN, Boston, MA) were added to each sample, mixed and incubated for 16-20 hours at 4°C. 200 µl of charcoal/dextran mixture was added to each tube, vortexed and incubated at 4°C for 20 minutes. Samples were centrifuged at 2000xg for 30 minutes and poured into scintillation tubes containing 5 mL of Ecolume biodegradable scintillation cocktail (cat# 882470; MP Biomedicals, Irvine, CA). Samples were counted for 1 minute using a Beckman beta counter. Unknown samples were analyzed using AssayZap (Biosoft Inc., Cambridge, UK) to calculate concentrations of unknowns in comparison to a known standard curve.

*Western Blot*

StAR protein and cytochrome P450 side-chain cleavage (P450scc) enzyme protein expressions were measured in the same normal and poor semen quality stallions using western blot techniques (see Appendix G for protocol). StAR and P450scc proteins were measured in freshly frozen testis tissue as well as from testicular cells cultured with *in vitro* hCG and forskolin treatments. Proteins were extracted using Tris-Sucrose-EDTA homogenization fluid. Protein samples were run on a 12.5% polyacrylamide gel and transferred to a nitrile membrane. The membrane was blocked with blocking buffer, probed with StAR primary antibody (Dr. Stocco, Texas Tech University, TX) and anti-rabbit IgG secondary antibody (cat# NA934; Amersham Biosciences, Piscataway, NJ). The membrane was developed with chemiluminescence reagents (NEN, Boston, MA) and exposed to X-ray film for band visualization. The same membranes were also

stripped and re-probed with P450scc primary antibody (cat# AB1244; Chemicon International, Inc., Temecula, CA) and the same secondary antibody used previously. Chemiluminescence reagents were used to develop membranes for exposure to X-ray film (cat# RPN 1678 H, Hyperfilm MP; Amersham Pharmacia, Piscataway, NJ).

**Results**

*Semen Quality Evaluation*

Left and right testicular parenchymal weight (Figure 36) and total testicular parenchymal weight (Figure 37) was significantly different ($P<0.05$) between normal and poor semen quality stallions. Left and right testis daily sperm production (DSP) per gram of parenchyma, mean DSP per gram of parenchyma, left and right testis DSP per testis and DSP per horse (Figures 38-41) were significantly different ($P<0.05$) between normal and poor semen quality stallions. Prior to castration, semen was collected to determine daily sperm output (DSO) and motility, progressive motility and morphology were evaluated. DSO, total motility, progressively motile sperm (PMS) and morphologically normal sperm (MNS) (Figures 42 to 45) were significantly different ($P<0.05$) between normal and poor semen quality stallions. Apoptosis quantification was performed by counting the number of TUNEL-positive germ cells per one hundred Sertoli cells for each stallion. Left and right testis apoptotic rate (Figure 46) and mean apoptotic rate (Figure 47) were significantly different ($P<0.05$) between normal and poor semen quality stallions

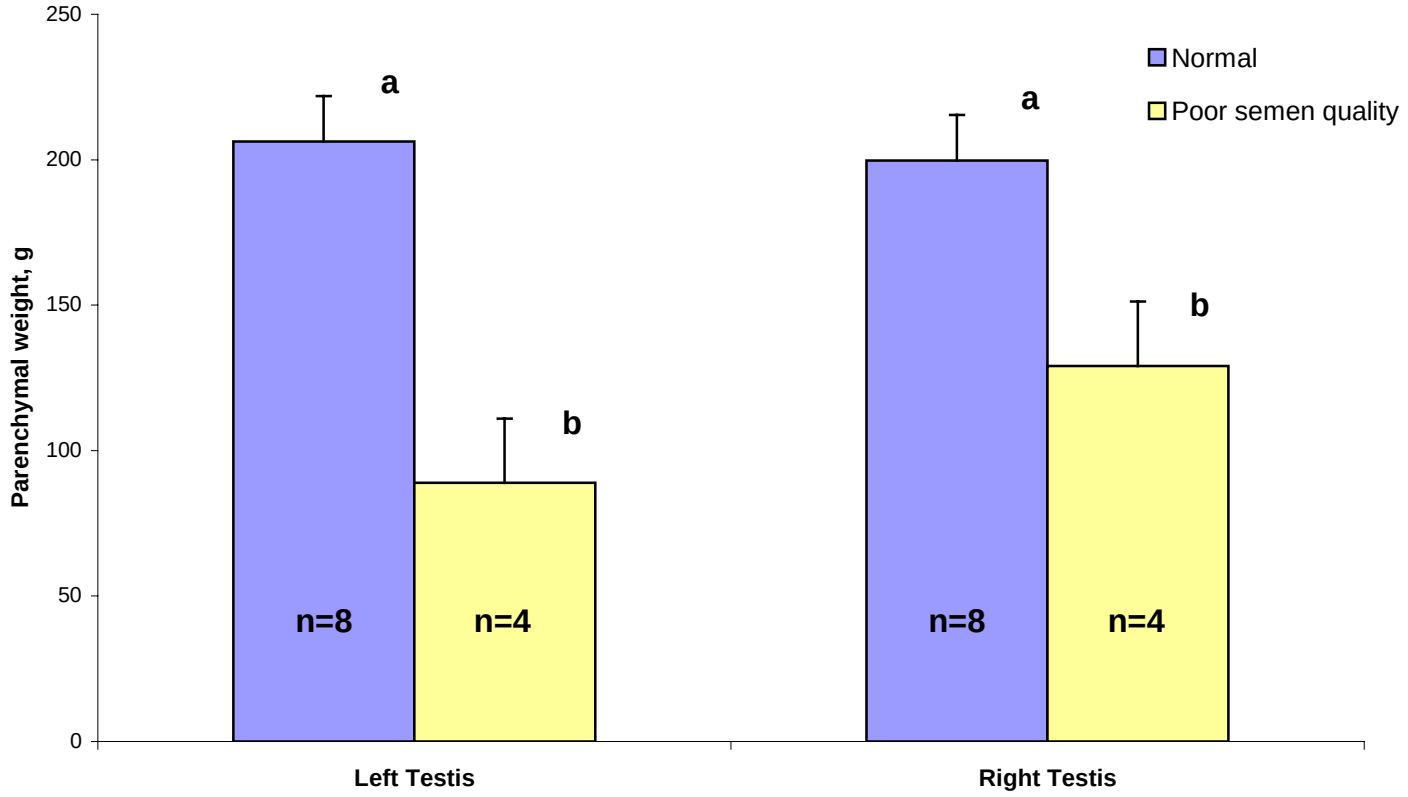


Figure 36. Left and right testicular parenchymal weight (g) for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

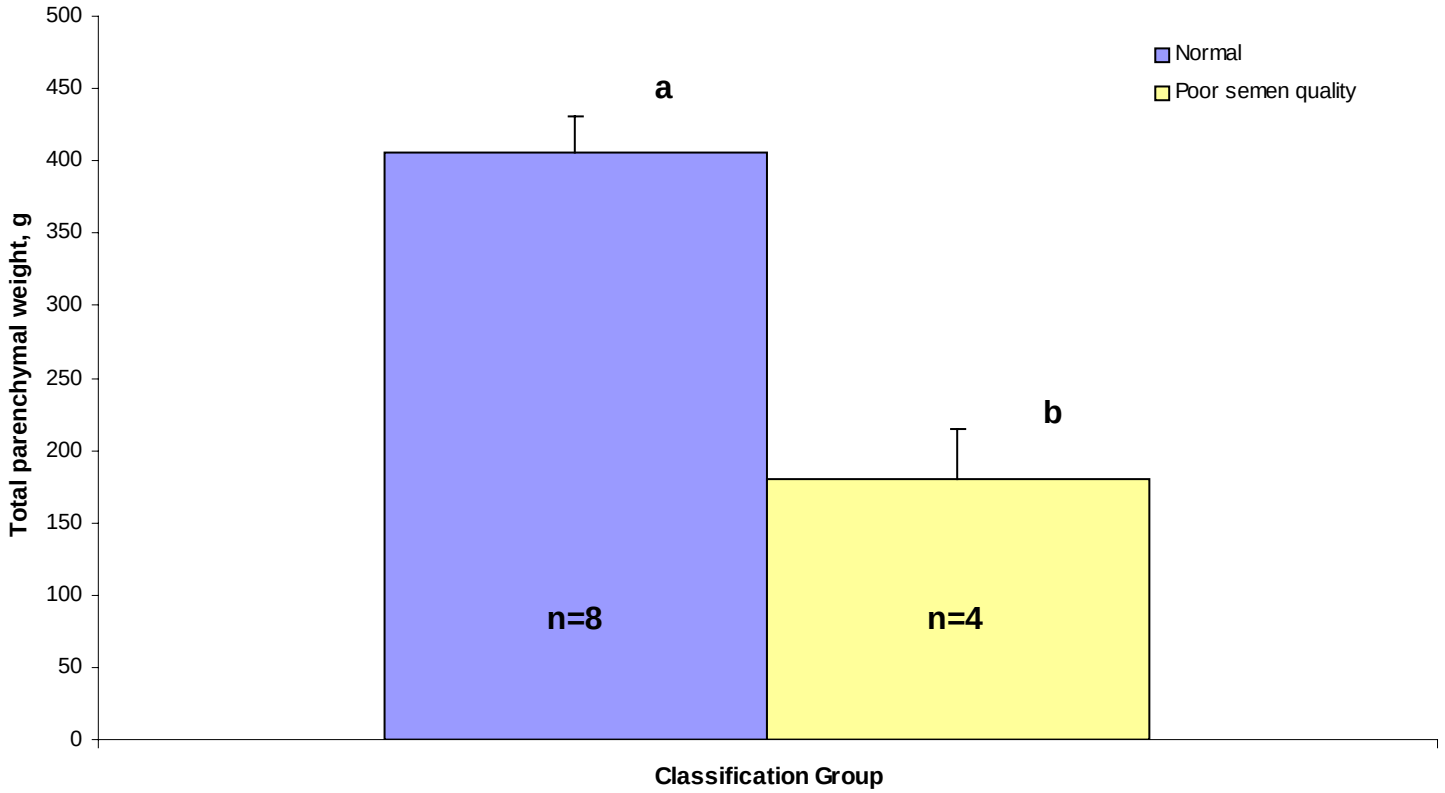


Figure 37. Total testicular parenchymal weight (g) for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

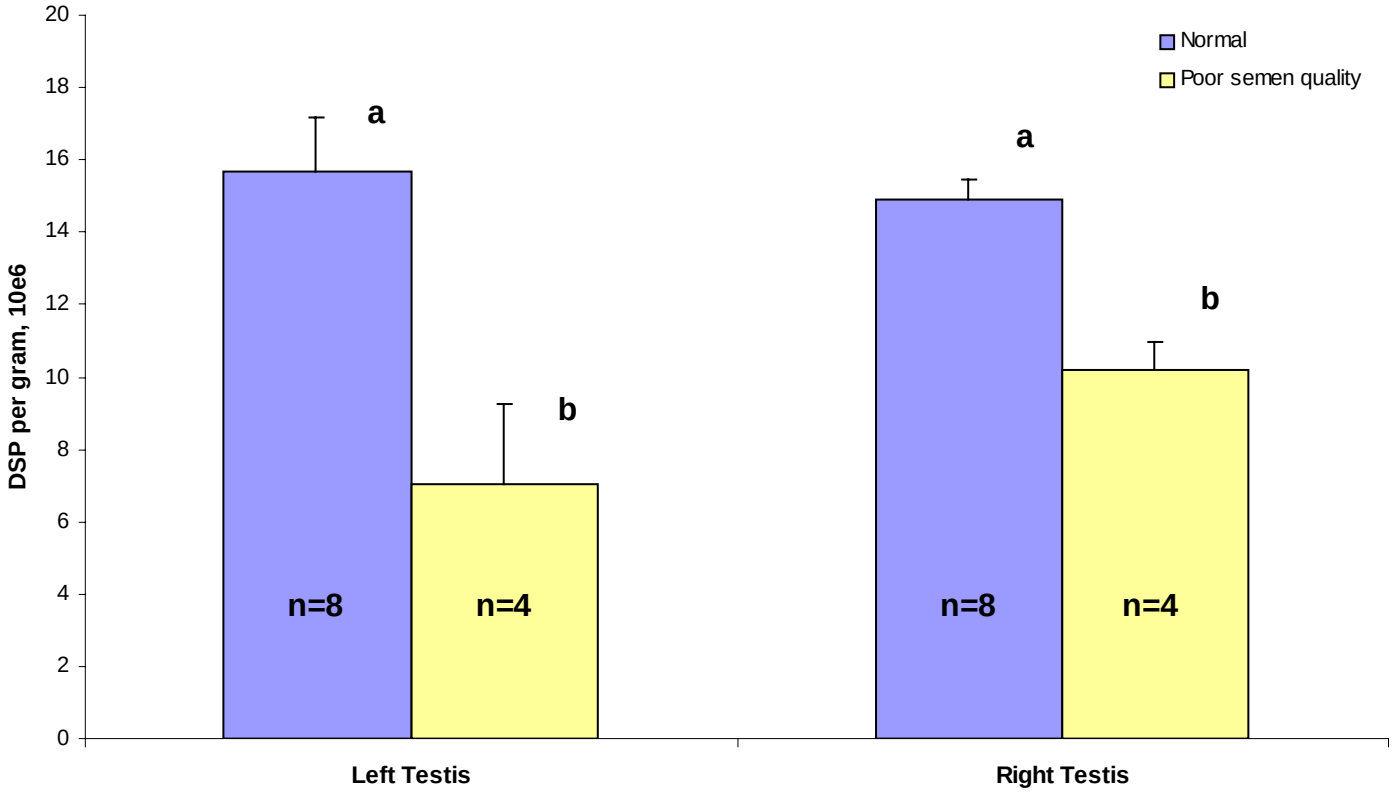


Figure 38. Left and right daily sperm production (DSP) per gram of parenchyma for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

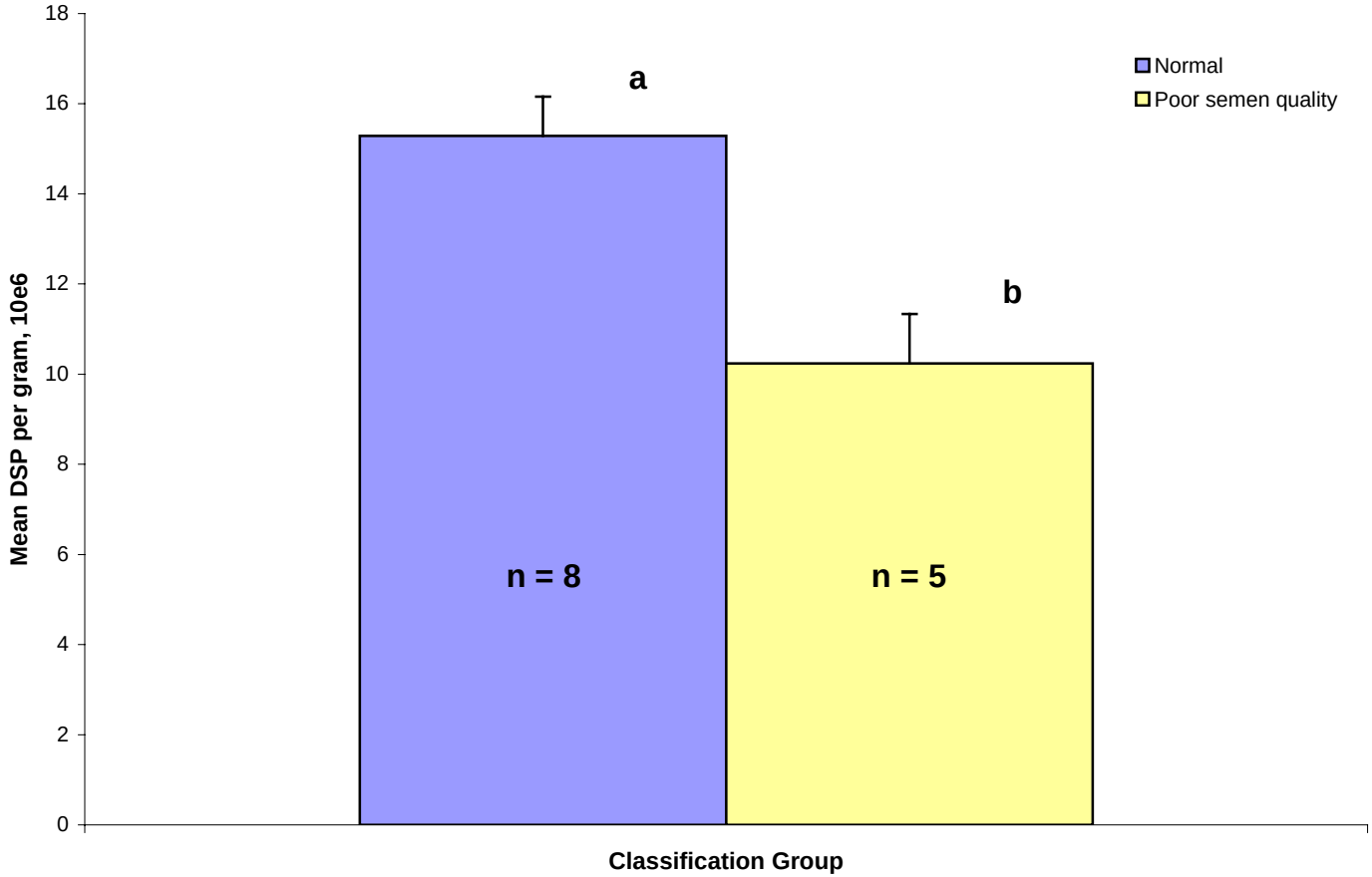


Figure 39. Mean daily sperm production (DSP) per gram of parenchyma for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

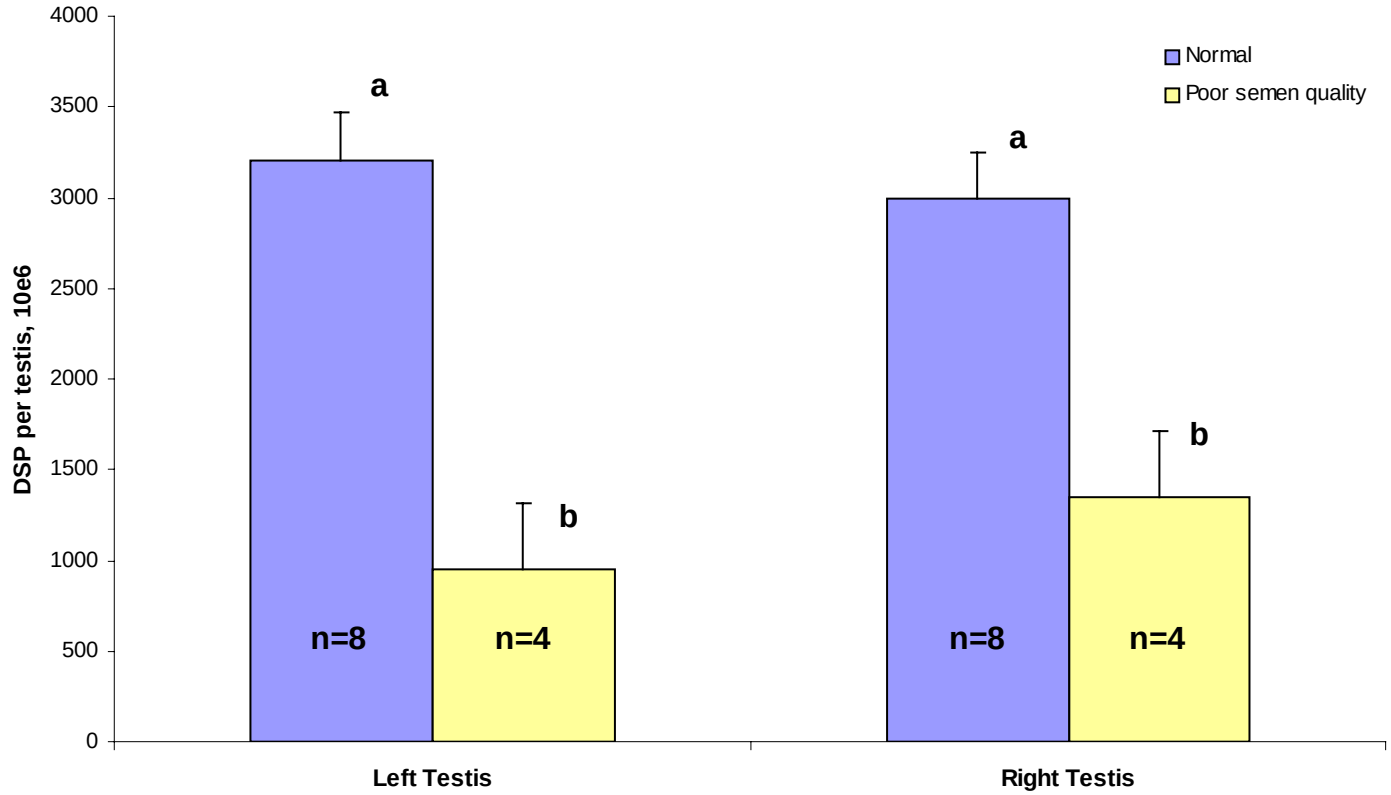


Figure 40. Left and right daily sperm production (DSP) per testis for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

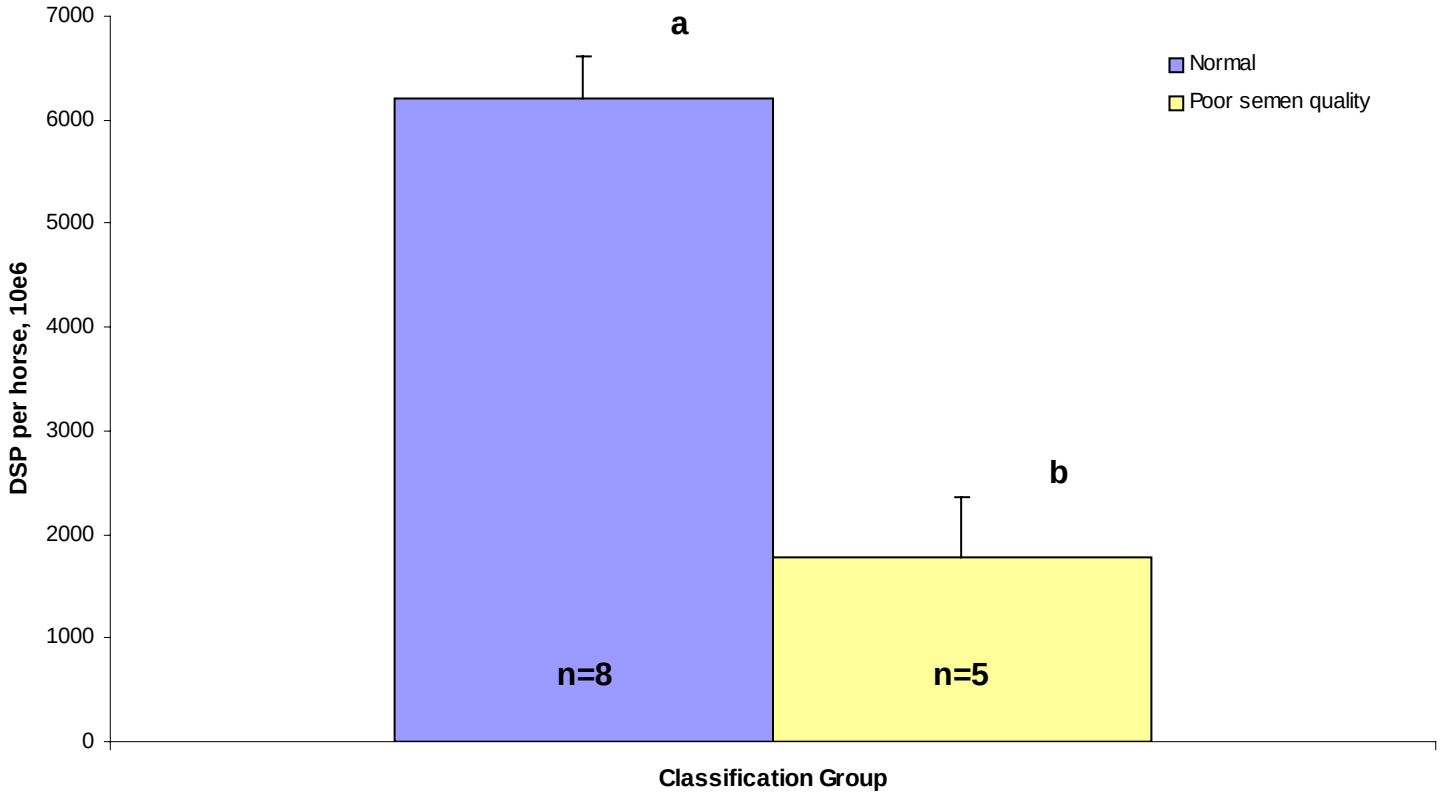


Figure 41. Daily sperm production (DSP) per horse for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

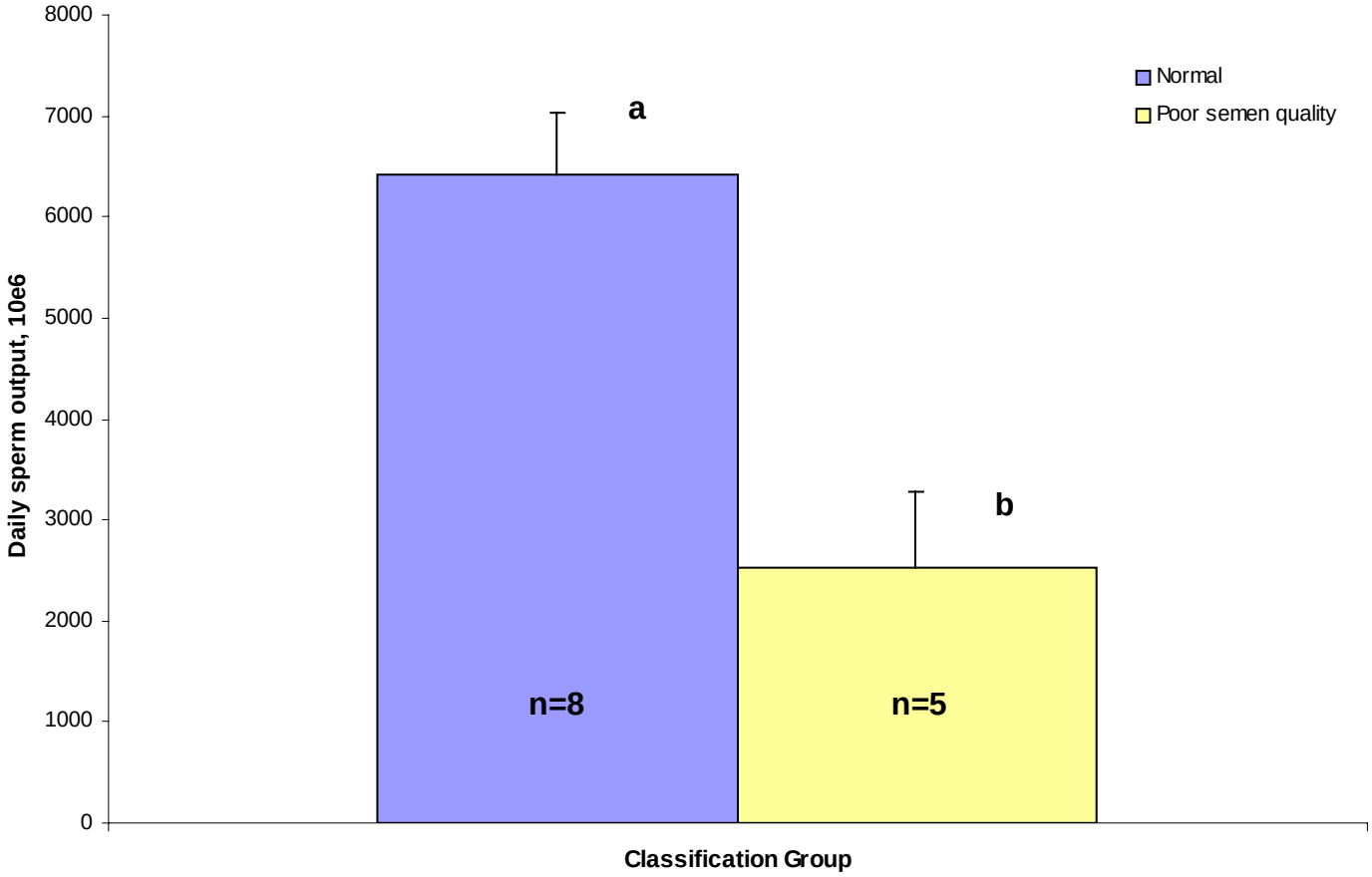


Figure 42. Daily sperm output (10e6) per horse for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

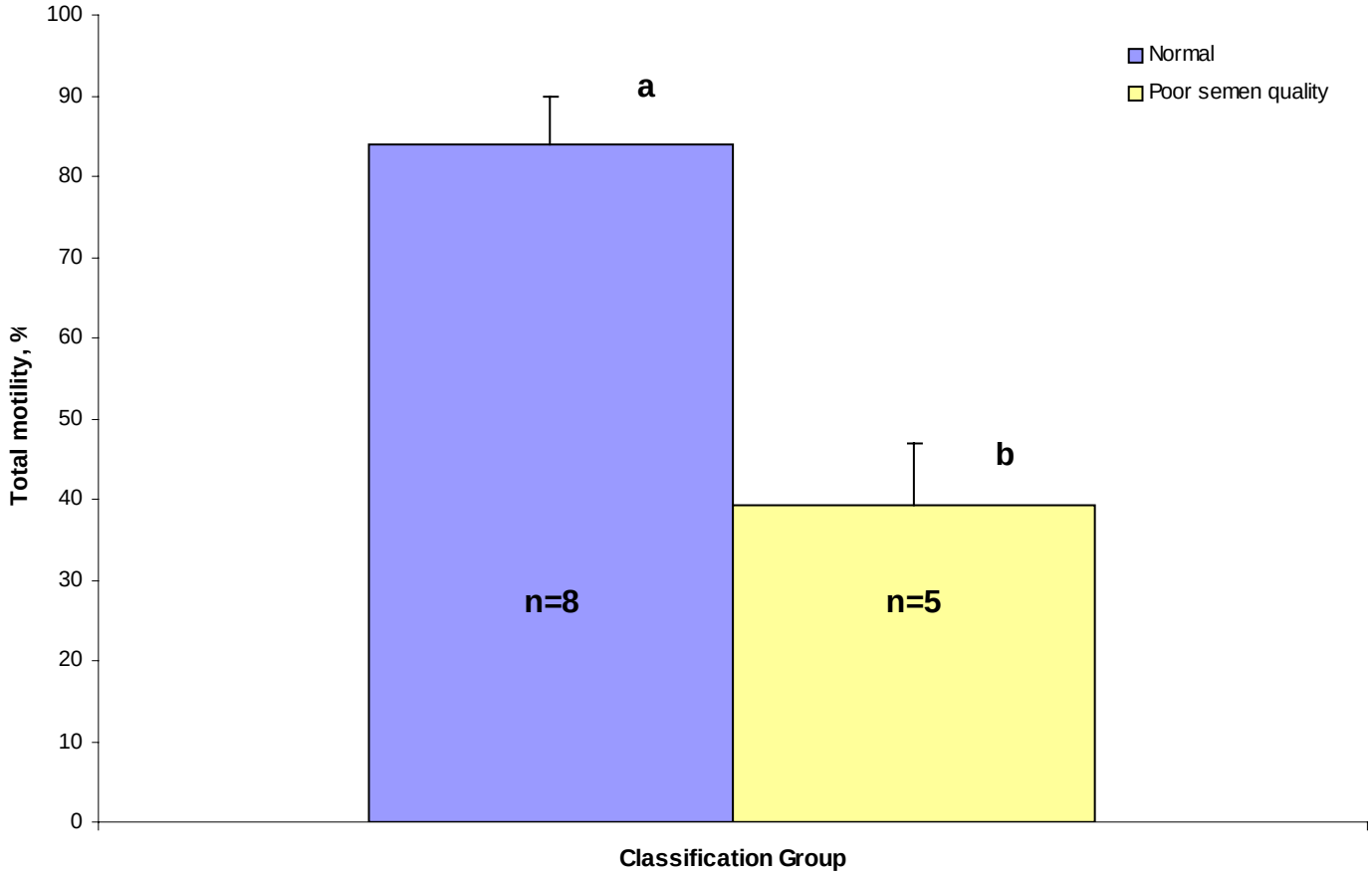


Figure 43. Total motility (%) per horse for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ ($P<0.05$).

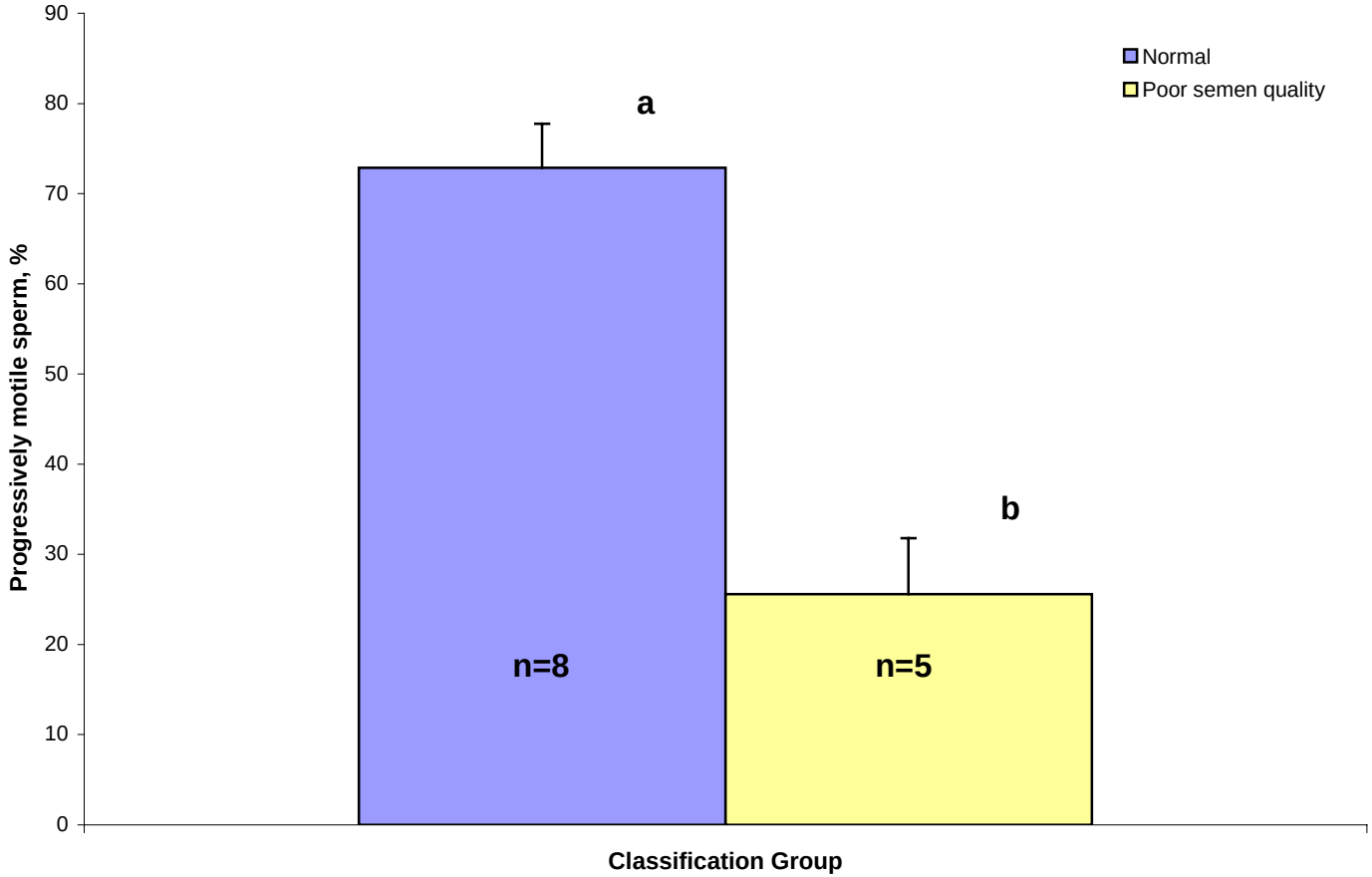


Figure 44. Progressively motile sperm (%, PMS) per horse for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

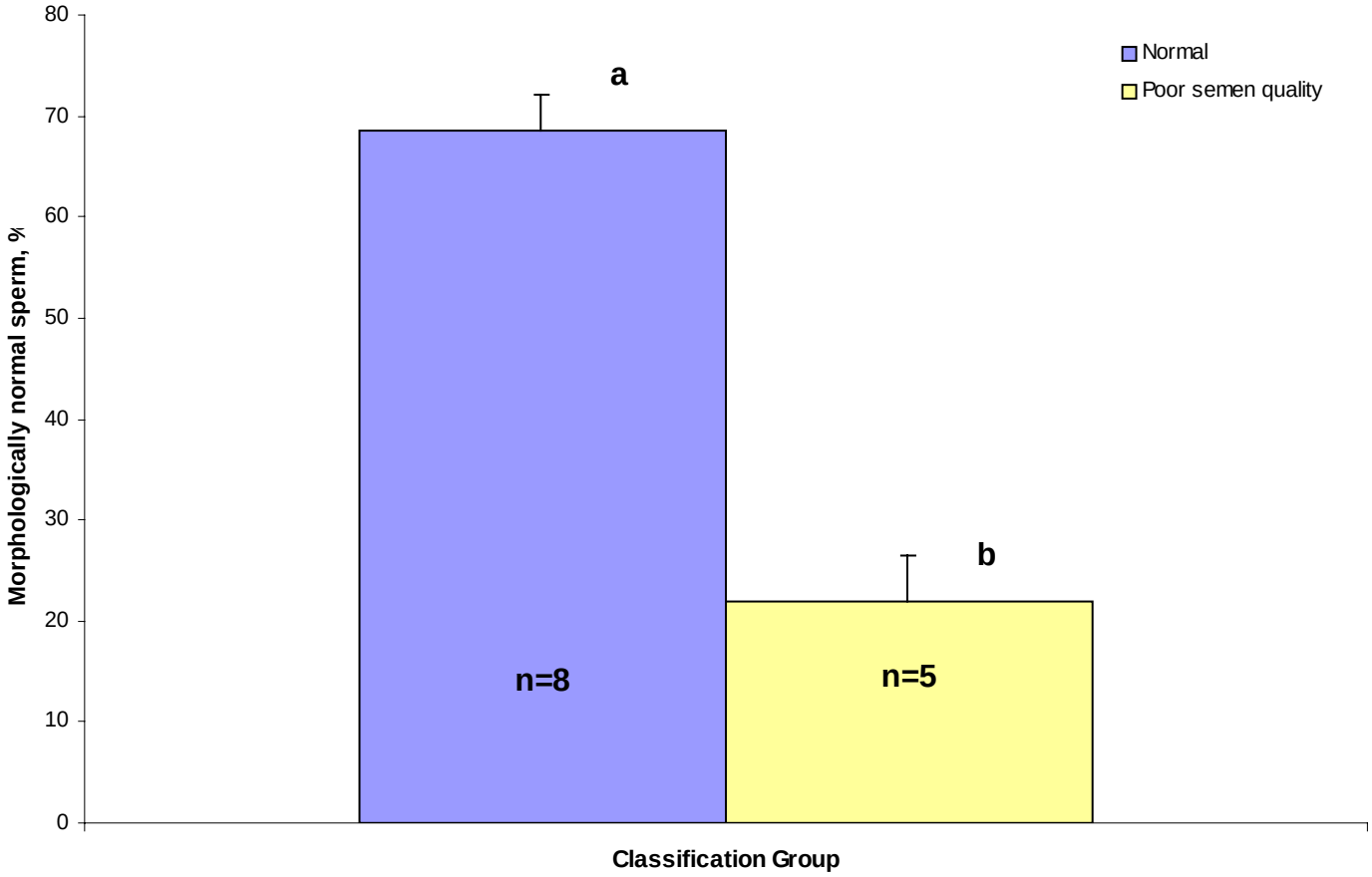


Figure 45. Morphologically normal sperm (%, MNS) per horse for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

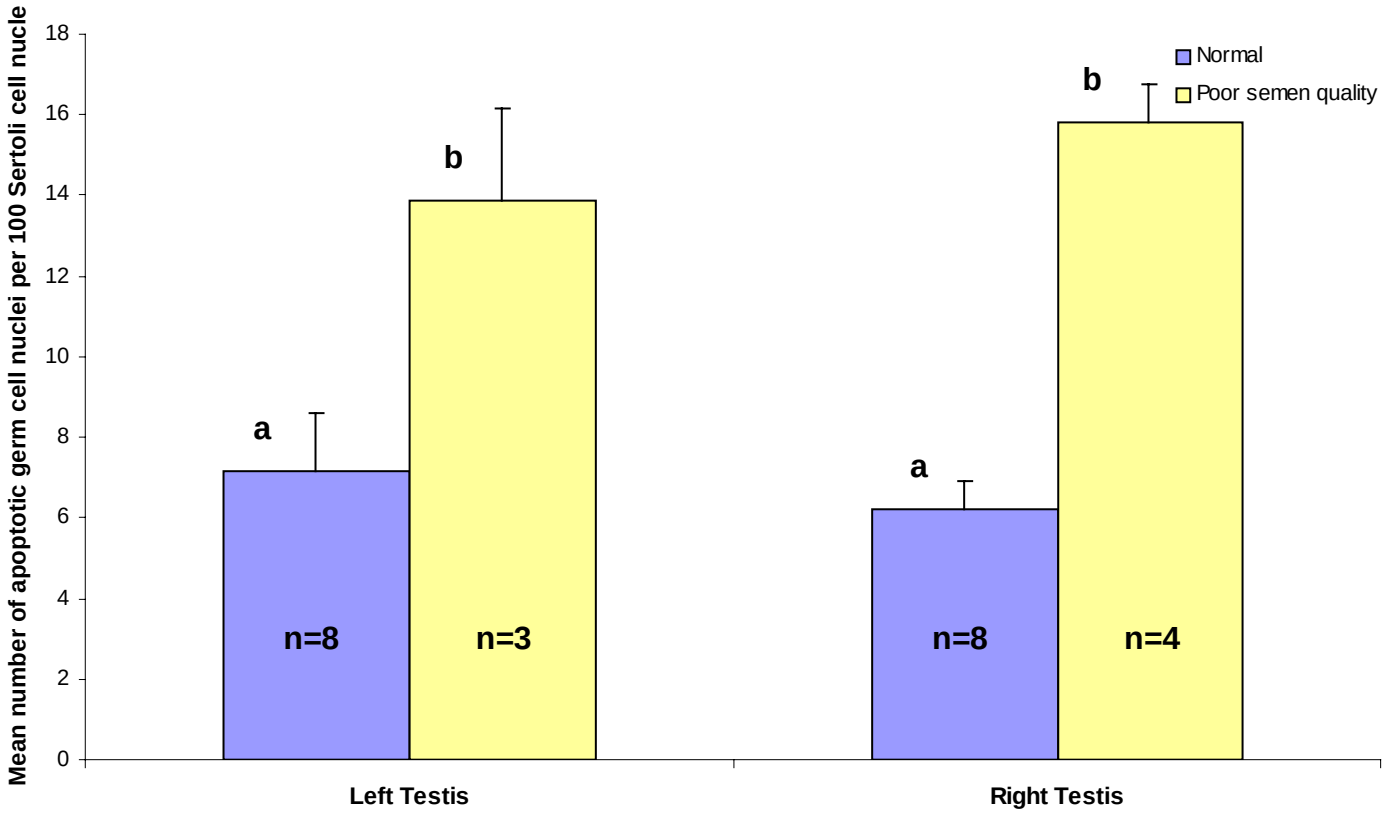


Figure 46. Left and right testis mean number of apoptotic germ cell nuclei per 100 Sertoli cell nuclei in normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ ($P<0.05$).

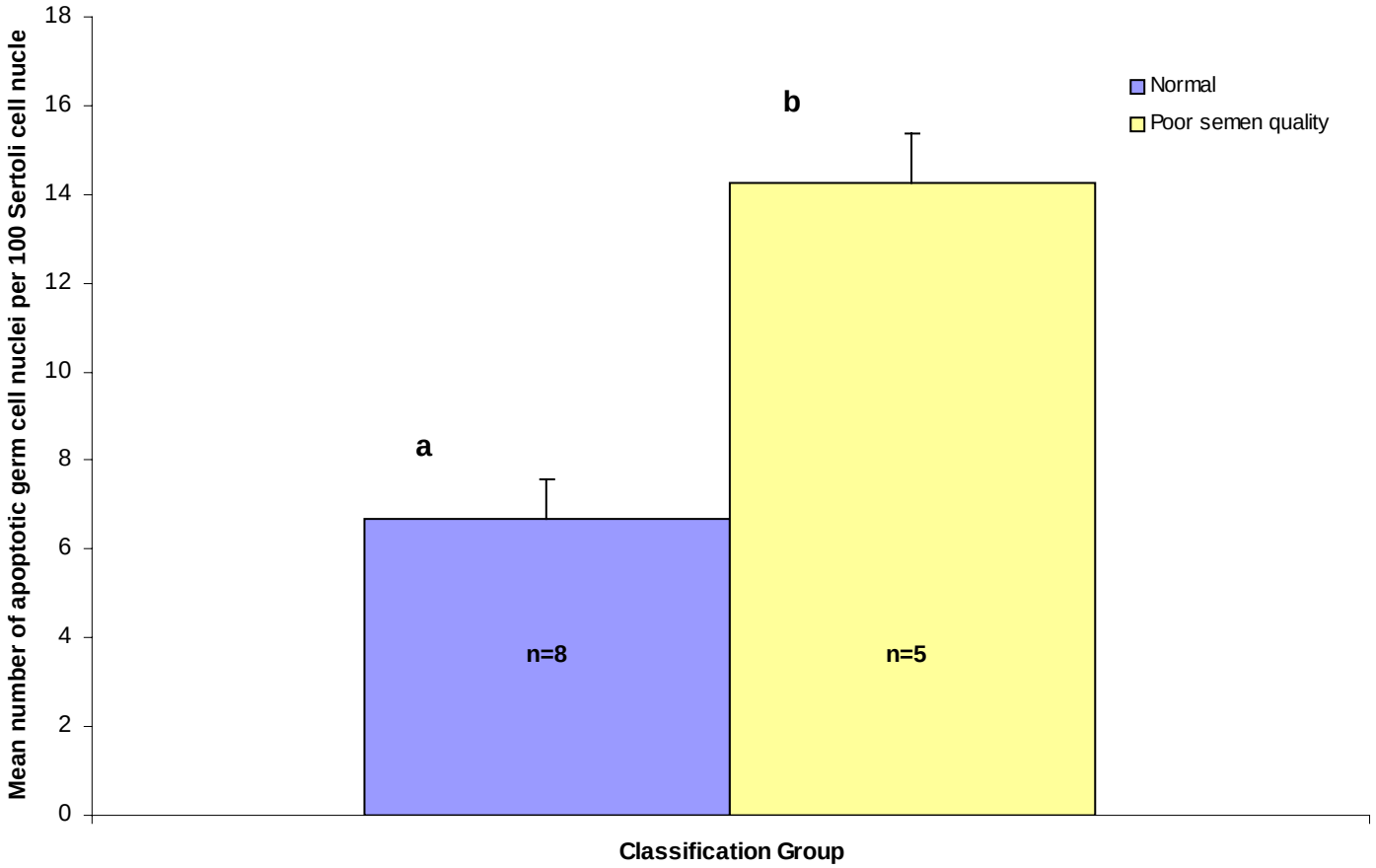


Figure 47. Mean number of apoptotic germ cell nuclei per 100 Sertoli cell nuclei in normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

*Hormone Assays*

Mean plasma testosterone and LH concentrations (Figures 48 and 49) were not different between normal and poor semen quality stallions. Mean plasma estradiol and inhibin concentrations (Figures 50 and 51) were significantly different ($P<0.05$) between normal and poor semen quality stallions. Mean plasma FSH concentration (Figure 52) had a tendency ($P<0.10$) to be higher in poor semen quality stallions than in normal stallions. Mean serum IGF-I concentration (Figure 53) had a tendency ($P<0.10$) to be lower in poor semen quality stallions than in normal stallions.

In Vitro *Culture*

Testosterone output with hCG treatment *in vitro* (Figure 54) did not differ between normal and poor semen quality stallions. The testosterone response ratio with forskolin treatment *in vitro* (Figure 55) had a tendency to be lower ($P<0.10$) in poor semen quality stallions than normal stallions. Testis content of StAR protein with hCG treatment *in vitro* (Figure 56) did not differ between normal and poor semen quality stallions.

*Western Blot*

Western blot analysis was used to measure testis content of StAR and P450scc proteins (Figures 57 and 58). Concentrations of the StAR and P450scc proteins were not different between normal and poor semen quality stallions (Figures 59 and 60).

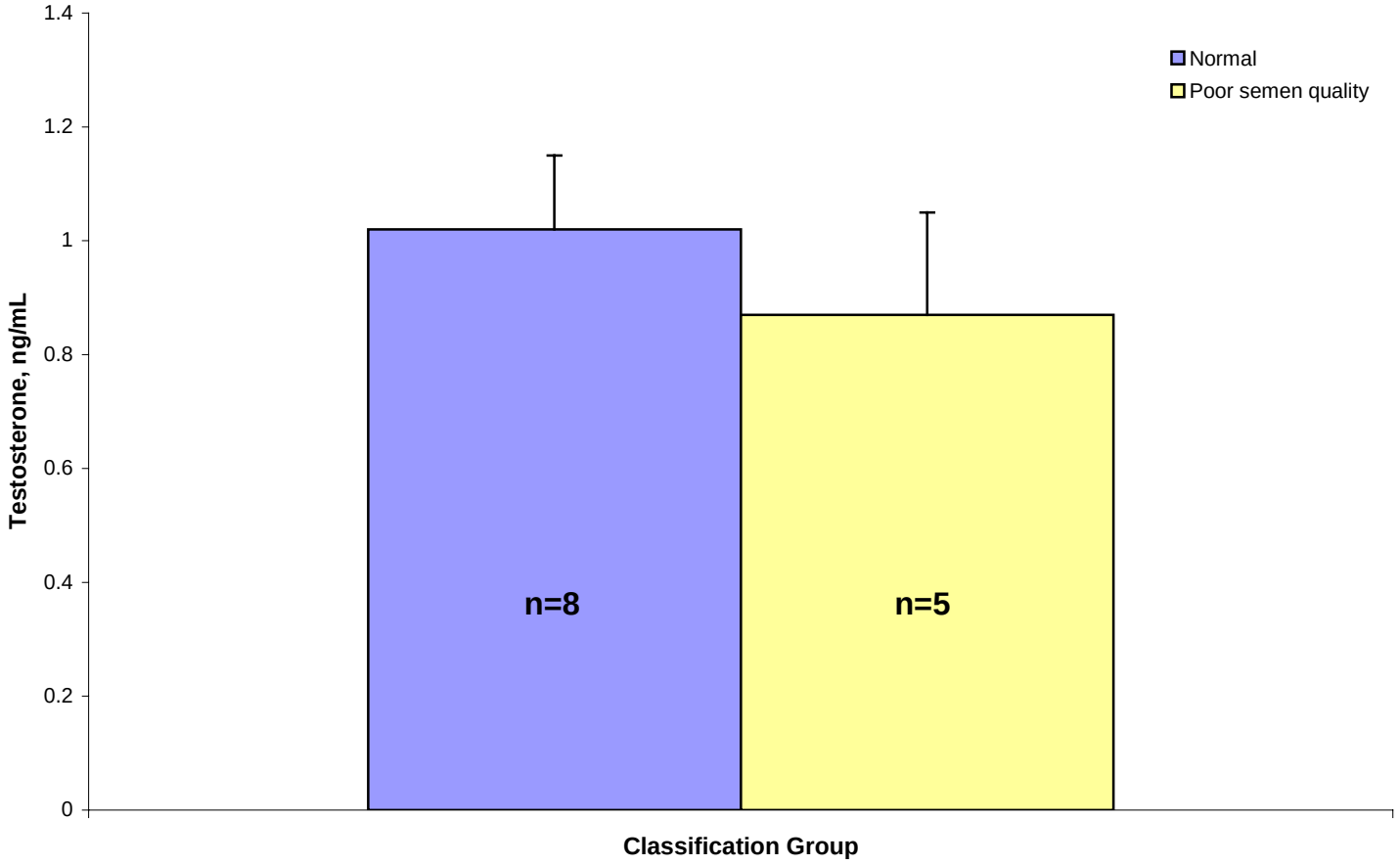


Figure 48. Mean plasma testosterone concentration (ng/mL) for normal and poor semen quality stallions (n = number of stallions per classification group). Means do not differ between treatment groups.

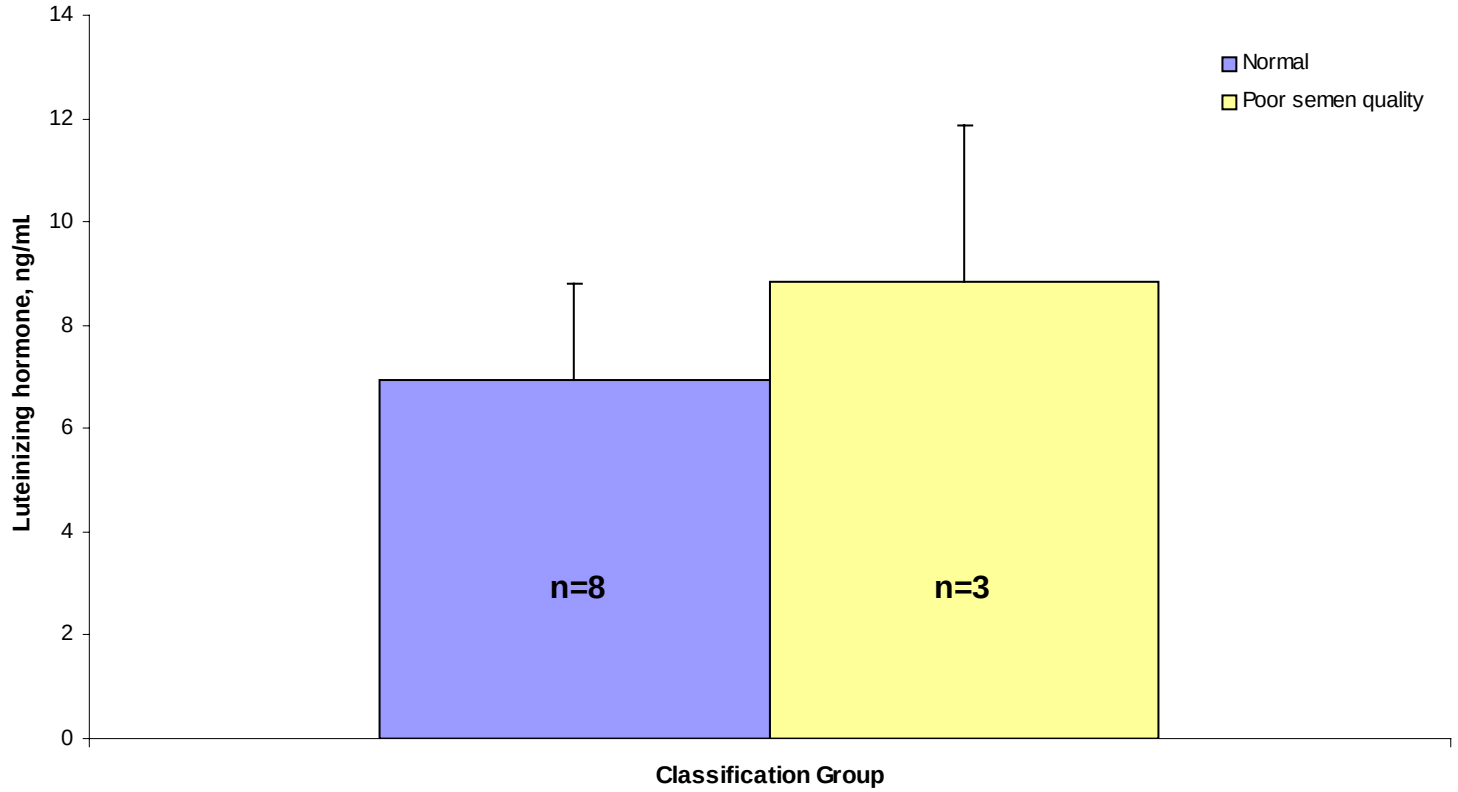


Figure 49. Mean plasma luteinizing hormone (LH) concentration (pg/mL) for normal and poor semen quality stallions (n = number of stallions per classification group). Means do not differ between treatment groups.

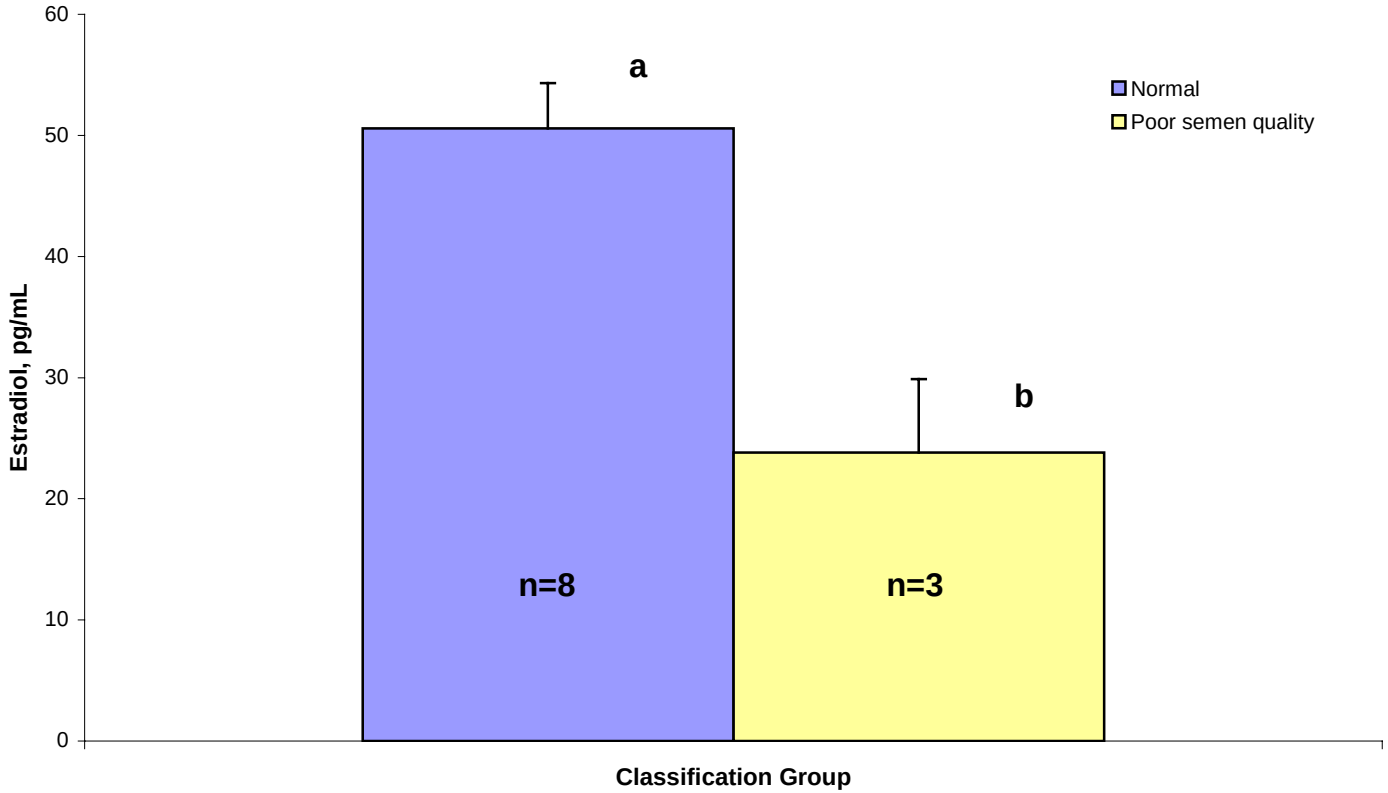


Figure 50. Mean plasma estradiol concentration (pg/mL) for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

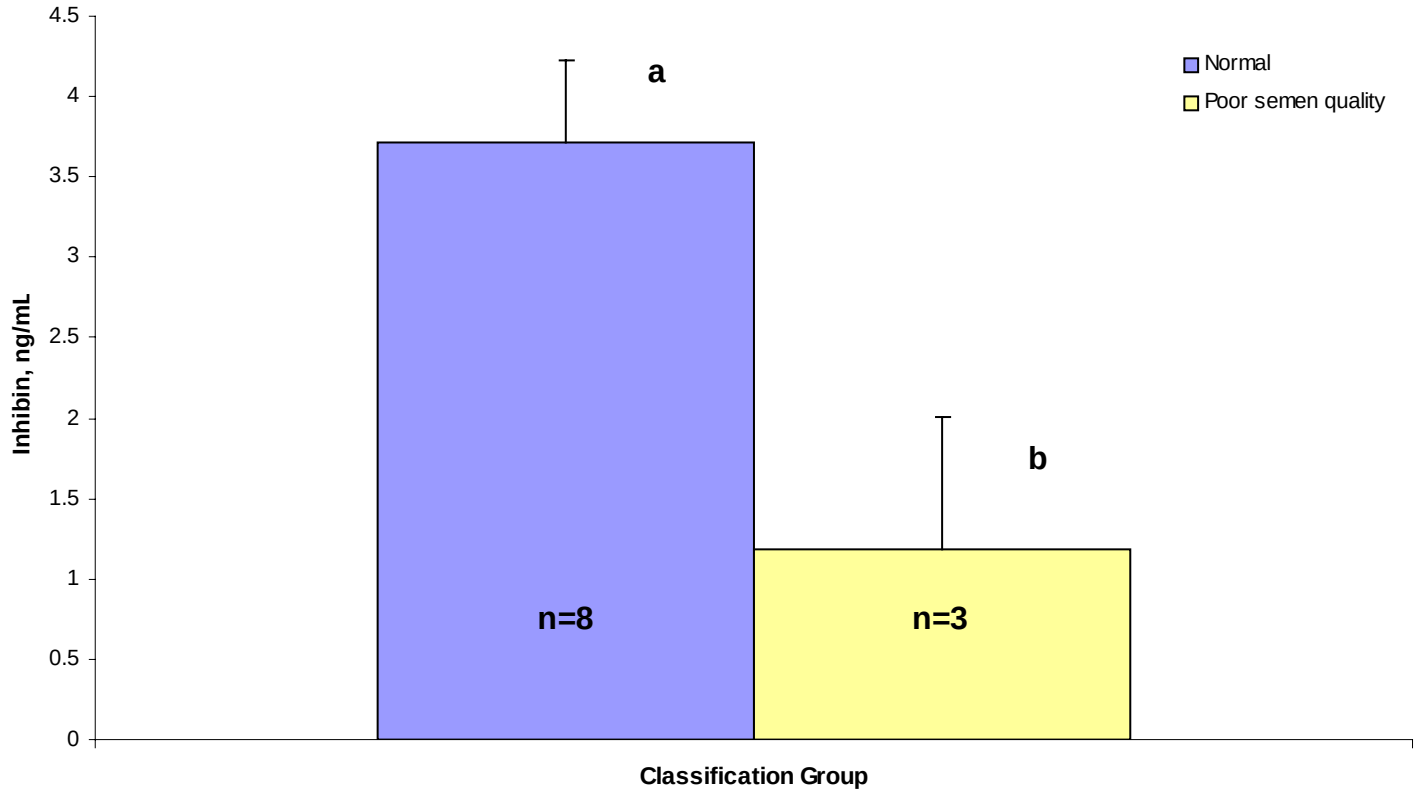


Figure 51. Mean plasma ir-inhibin concentration (ng/ml) for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.05).

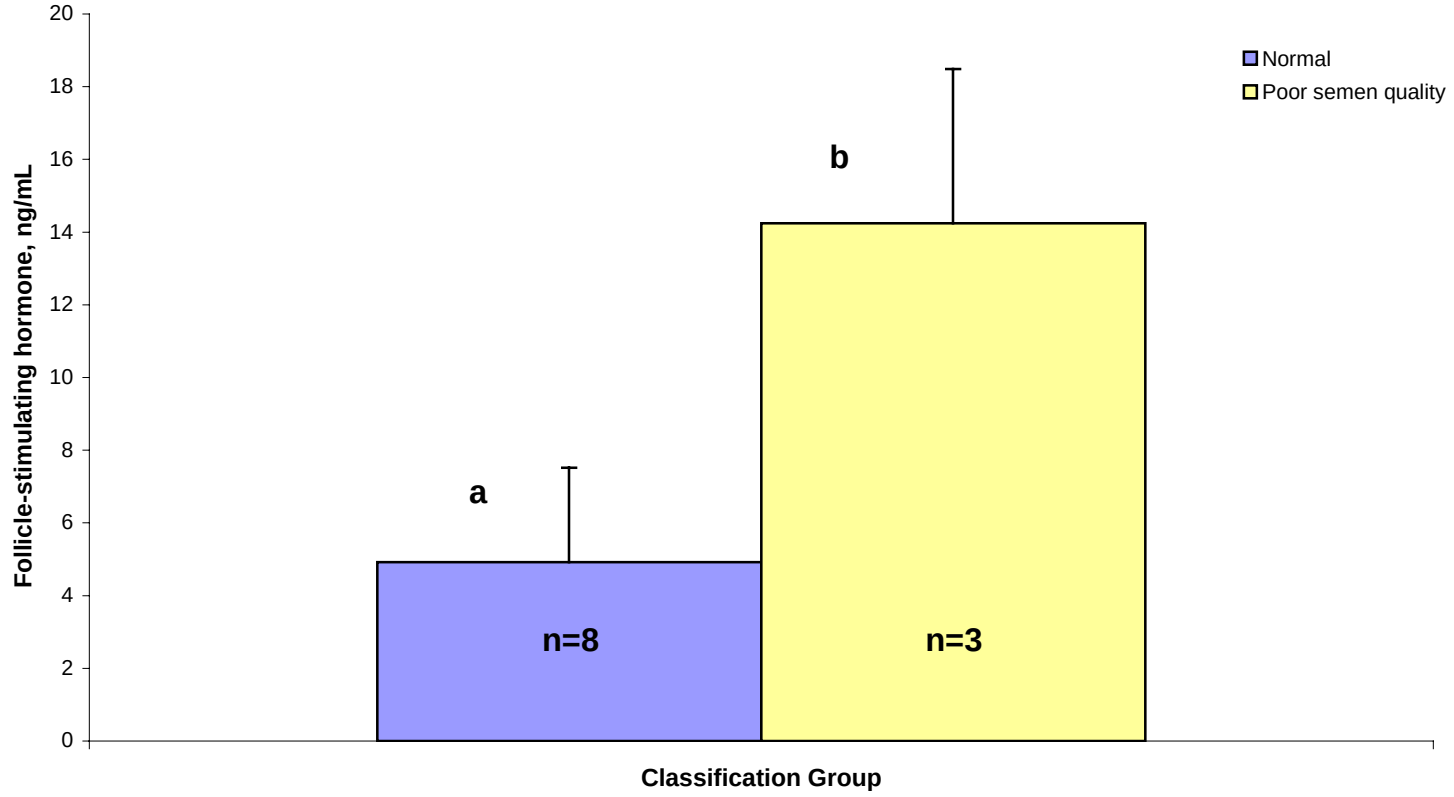


Figure 52. Mean plasma follicle-stimulating hormone (FSH) concentration (ng/ml) for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.10).

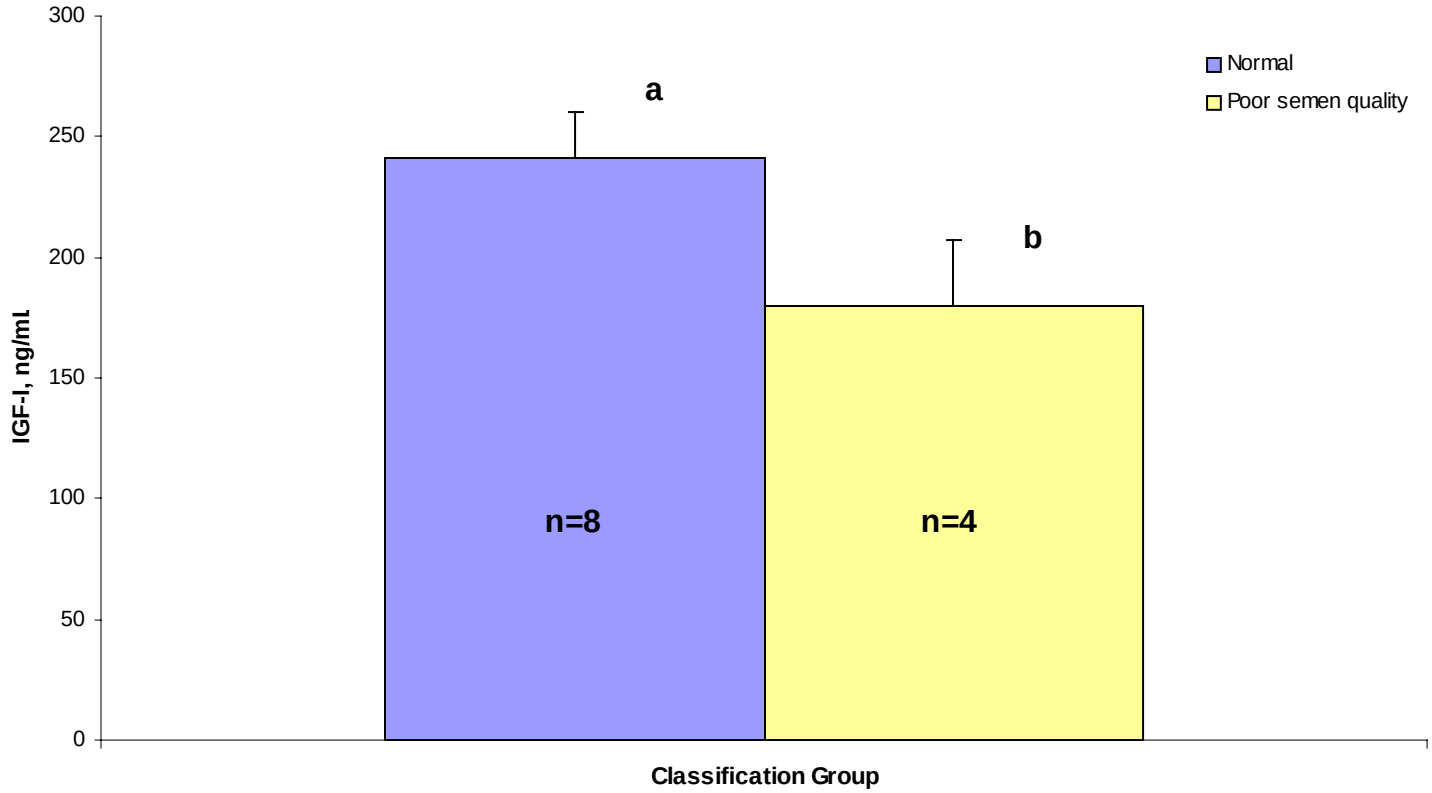


Figure 53. Mean serum insulin-like growth factor I (IGF-I) concentration (ng/ml) for normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.10).

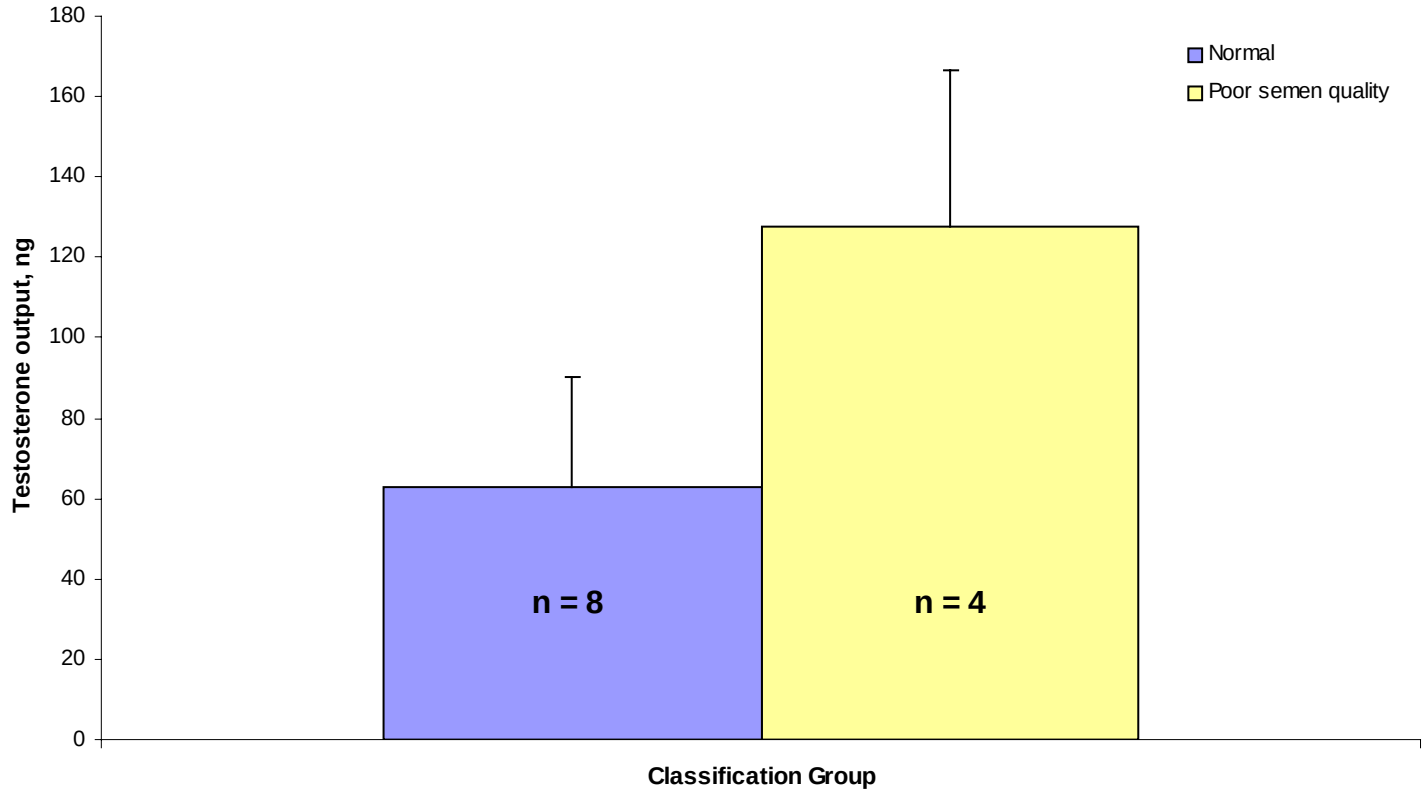


Figure 54. Testosterone output (ng) with hCG treatment *in vitro* in normal and poor semen quality stallions (n = number of stallions per classification group). Means do not differ between treatment groups (P>0.05).

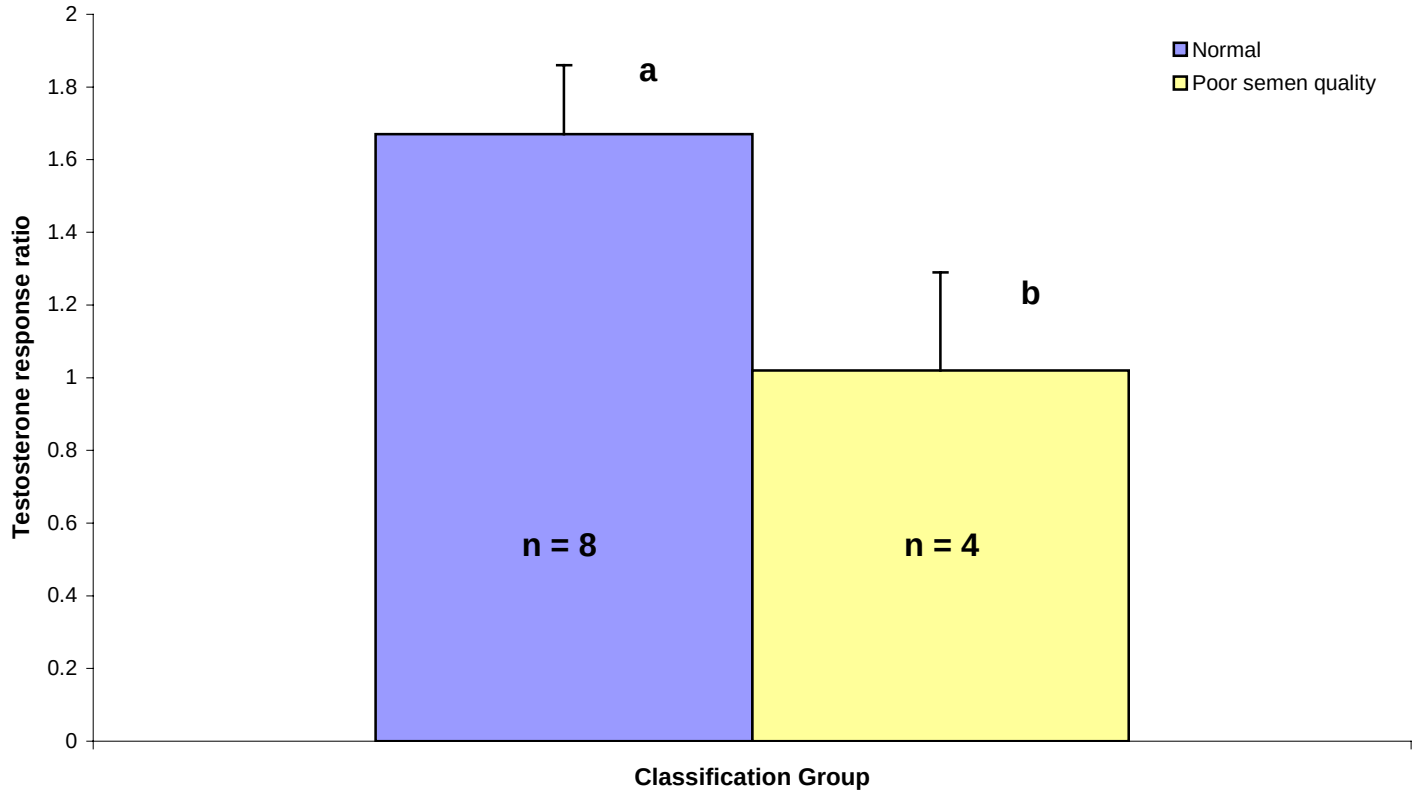


Figure 55. Testosterone response ratio with forskolin treatment *in vitro* in normal and poor semen quality stallions (n = number of stallions per classification group). Means with different subscripts differ (P<0.10).

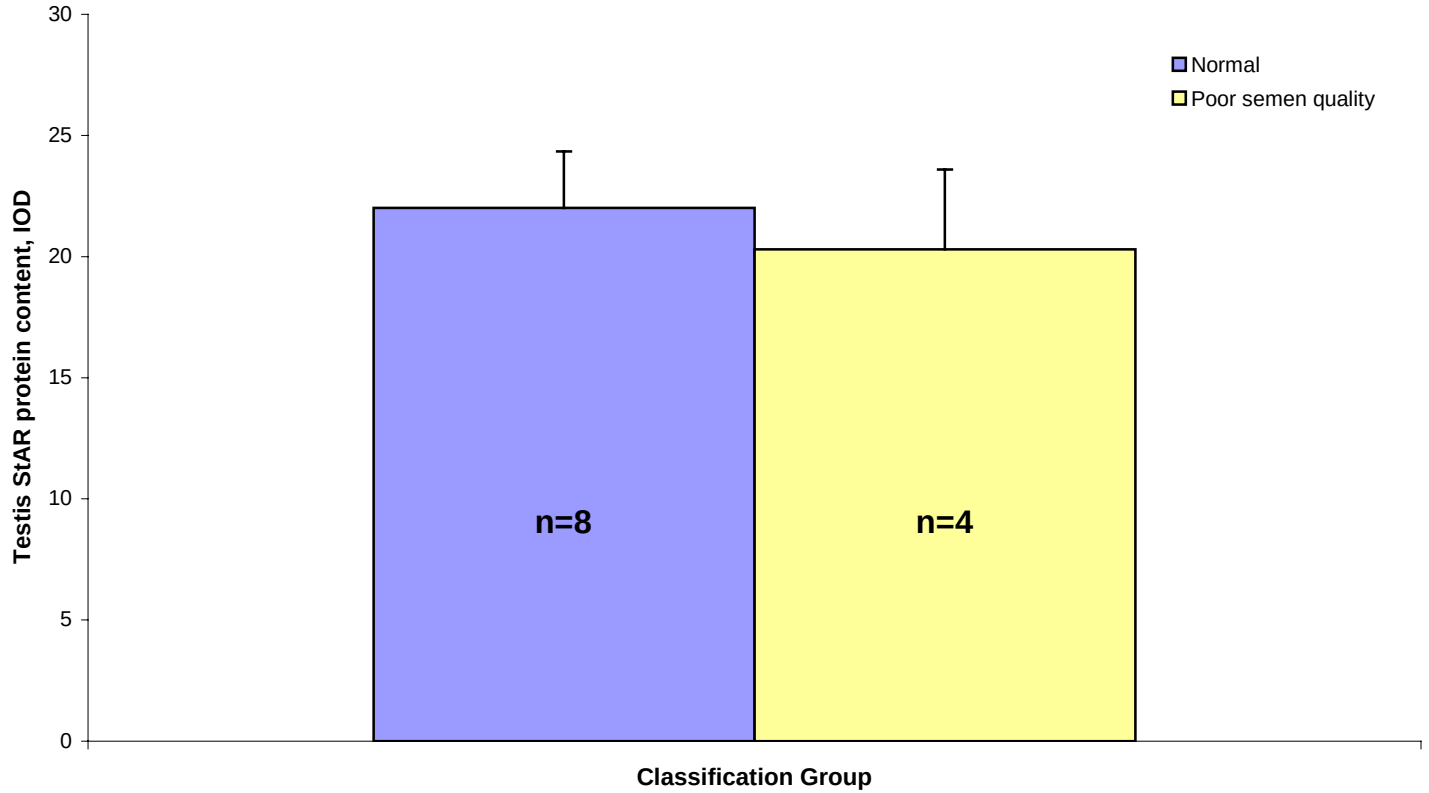


Figure 56. Testis content of StAR protein (IOD) with hCG treatment *in vitro* in normal and poor semen quality stallions (n = number of stallions per classification group). Means do not differ between treatment groups (P>0.05).

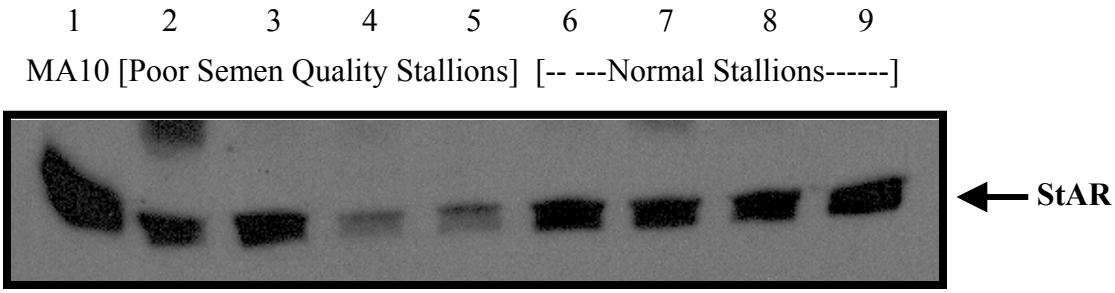


Figure 57. Western blot analysis of steroidogenic acute regulatory (StAR) protein expression in the testis of normal and poor semen quality stallions. Lane 1 contains the 30-kDa StAR protein positive control consisting of MA10 cells cultured from the mouse MA10 Leydig cell line. Lanes 2 through 5 demonstrate StAR content in four different poor semen quality stallions while lanes 6 through 9 demonstrate StAR content in four different normal stallions.

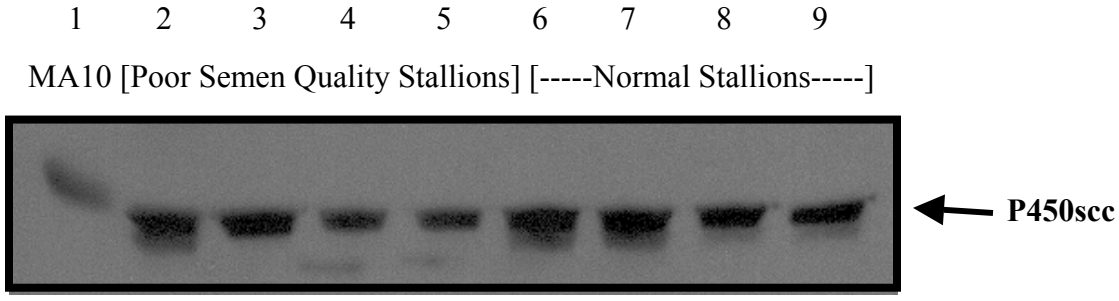


Figure 58. Western blot analysis of cytochrome P450 side-chain cleavage enzyme (P450scc) protein expression in the testis of normal and poor semen quality stallions. Lane 1 contains the 47-kDa P450scc positive control consisting of MA10 cells cultured from the mouse MA10 Leydig cell line. Lanes 2 through 5 demonstrate P450scc content in four different poor semen quality stallions while lanes 6 through 9 demonstrate P450scc content in four different normal stallions.

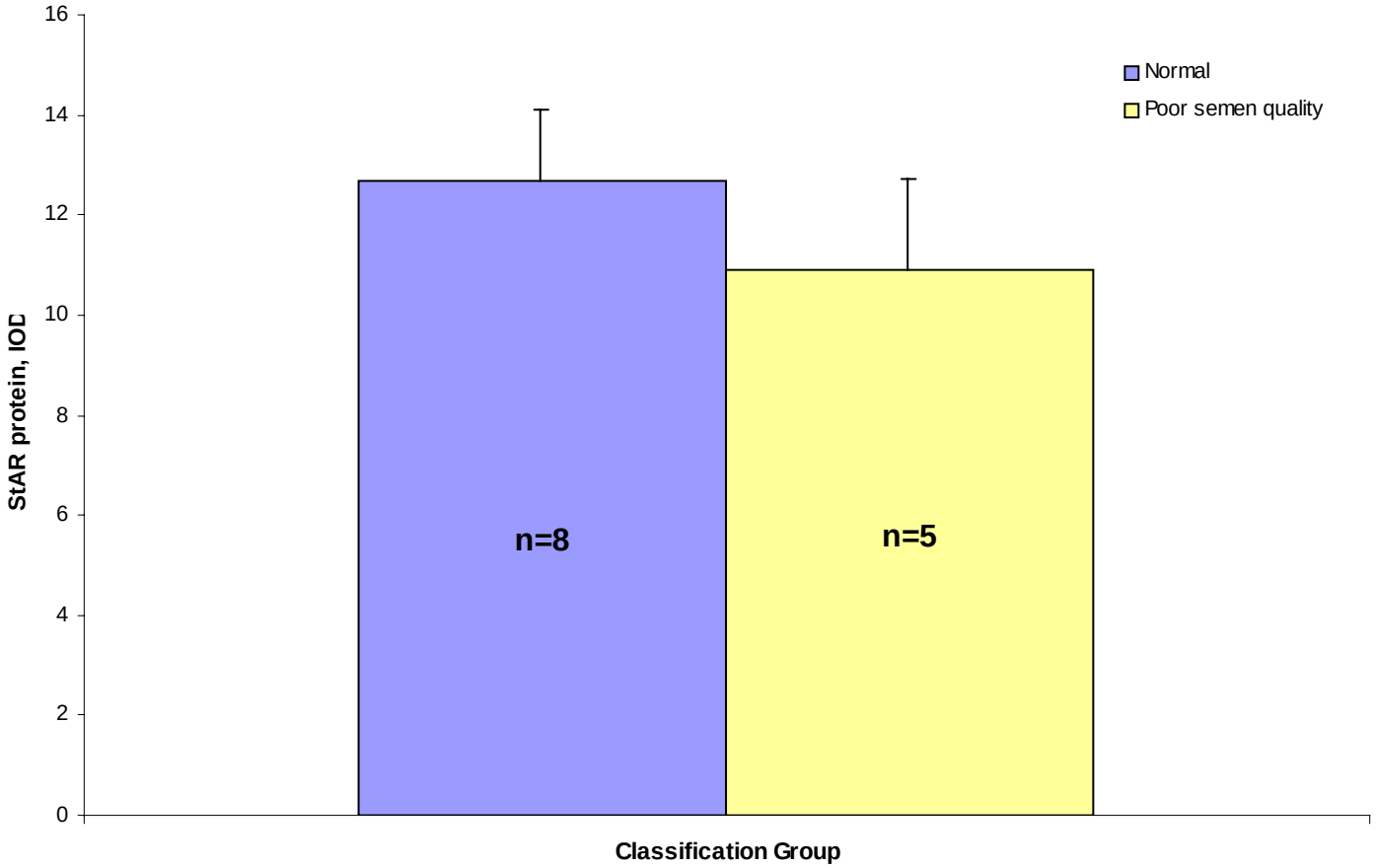


Figure 59. Testis tissue content of StAR protein (IOD) in normal and poor semen quality stallions (n = number of stallions per classification group). Means do not differ between treatment groups (P>0.05).

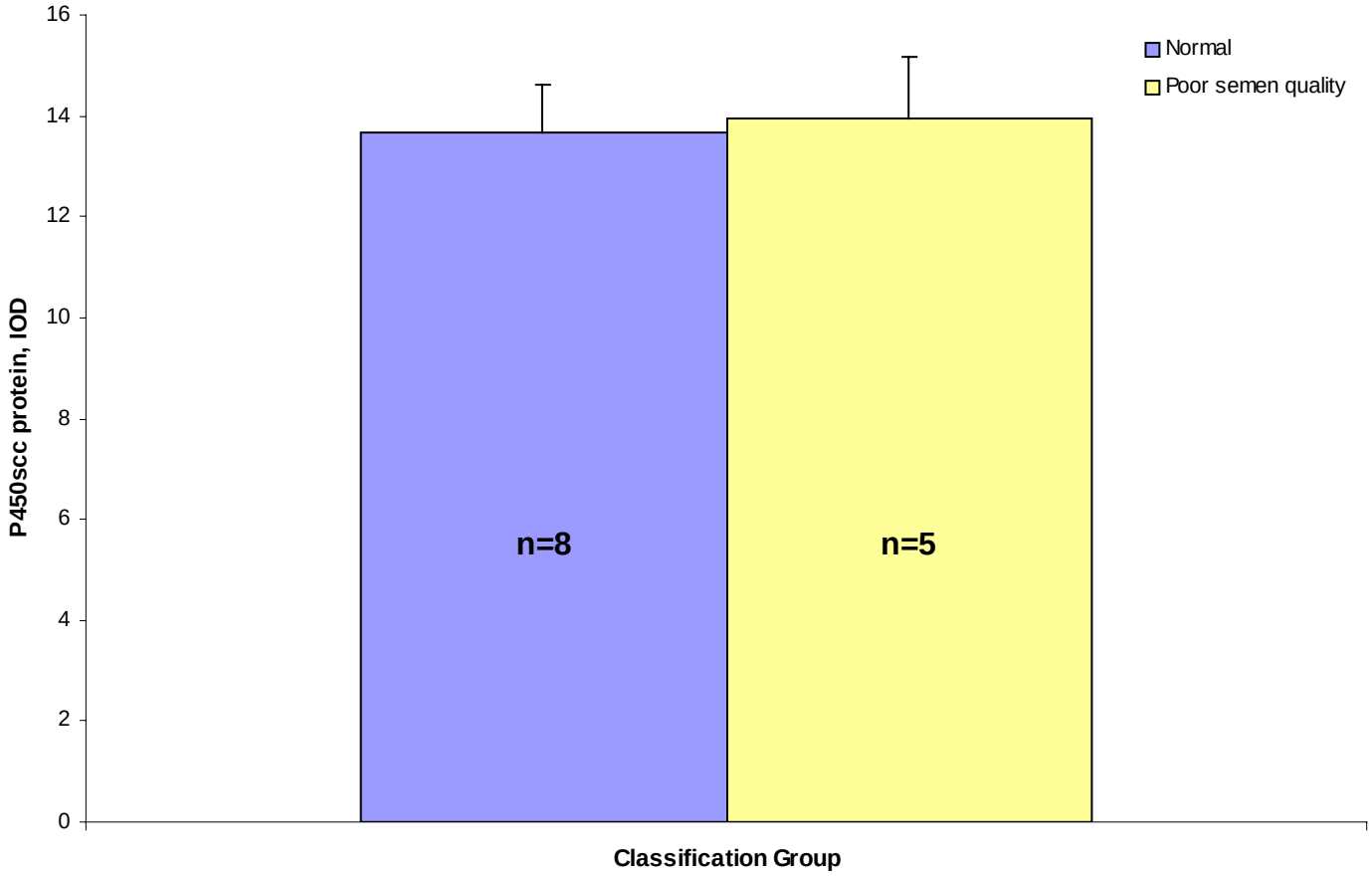


Figure 60. Testis tissue content of P450scc protein (IOD) in normal and poor semen quality stallions (n = number of stallions per classification group). Means do not differ between treatment groups (P>0.05)

**Discussion**

The parameters that reflect endocrine and gametogenesis function of the testis consistently demonstrated statistical differences between the normal group and poor semen quality group of stallions. The testicular parenchymal weights were statistically different between the normal and poor semen quality stallions. Also, all DSP measurements were significantly different between normal and poor semen quality stallions. Previously, a high correlation was reported between parenchymal weight and DSP (Johnson et al., 1994). Similarly, in this study total parenchymal (PAR) weight (WT) was highly correlated with mean DSP per gram (DSP/g) PAR WT ($r=0.7560$; $P<0.0044$) and DSP per horse (DSPH) ($r=0.9640$; $P<0.0001$). Left testis (LT) PAR WT was correlated with LT DSP/g ($r=0.8089$; $P<0.0014$) and LT DSP per testis (DSPT; $r=0.95101$; $P<0.0001$). Right testis (RT) PAR WT was positively correlated with RT DSP/g ($r=0.6952$; $P<0.0121$) and RT DSPT ($r=0.9402$; $P<0.0001$).

DSO, motility, PMS and MNS were significantly different between normal and poor semen quality stallions. These parameters of spermatogenesis indicate that the stallions in the poor semen quality group had testicular abnormalities leading to small testis size and low sperm production. The sperm that were collected in the poor semen quality group also had problems with motility and morphology. DSO, motility, PMS and MNS were all correlated with the following: LT PAR WT, LT DSP/g, LT DSPT, RT PAR WT, RT DSP/g, RT DSPT, total PAR WT, DSPH and Mean DSP/g ($r=0.6195$ to $r=0.9096$; $P<0.0001$ to $P<0.0317$) except PMS was not correlated with RT PAR WT. All of these data indicate that the size of the testes is related to sperm production, motility and morphology in the stallion.

Left and right testis apoptotic rate and mean apoptotic rate were significantly different between normal and poor semen quality stallions. LT apoptotic rate was inversely correlated with LT PAR WT (r=-0.6563; P<0.0283), LT DSPT (r=-0.6401; P<0.0339), total PAR WT (r=-0.6605; P<0.0376) and DSPH (r=-0.6788; P<0.0309). Similar inverse correlations were present for the RT. Mean apoptotic rate was inversely correlated with all PAR WT measurements and DSP parameters. The mean apoptotic rate was also inversely correlated with DSO (r=-0.7375; P<0.0040), motility (r=-0.6279; P<0.0216), PMS (r=-7057; P<0.0070) and MNS (r=-0.8768; P<0.0001). This information suggests that the amount of cell death that takes place in the stallion testis is a result of the size of the testes. Because of this, scrotal width measurements taken when performing BSE evaluations on stallions can be a useful tool in predicting apoptotic rate, DSP, DSO, motility, PMS and MNS. In order for this to be effective, future studies need to be performed to determine the relationship between scrotal width and parenchymal weight.

Mean plasma testosterone and LH concentrations were not different between normal and poor semen quality stallions. This means that circulating testosterone concentrations are not different due to the fertility of the animal and cannot be used as an indicator of fertility. Mean plasma FSH concentration had a tendency to be higher in poor semen quality stallions and mean plasma IGF-I had a tendency to be lower in poor semen quality stallions. First, Jan Roser's lab reported an increase in LH and FSH concentration in subfertile stallions (Roser and Hughes, 1992) then no difference in FSH concentration due to subfertility (Motton and Roser, 1997), however they indicated that plasma IGF-I concentration was directly related to declined fertility in stallions (Hess and

Roser, 2001). Further studies are necessary in order to determine if these hormones are different as a result of poor semen quality or if they are just due to the low number of stallions used in this study.

Mean plasma estradiol and immunoreactive (ir)-inhibin concentrations were significantly lower in poor semen quality stallions than in normal stallions. The decrease of estradiol found in poor semen quality stallions is contrary to previous work by Jan Roser; however, decline in ir-inhibin in subfertile stallions was noted in previous studies (Motton and Roser, 1997). Other investigators have indicated that estradiol is an important hormone in the regulation of stallion reproductive function (Roser, 2001). These results would support that hypothesis. Plasma estradiol concentration was correlated with total PAR WT ($r=0.8641$; $P<0.0013$), DSPH ($r=0.8047$; $P<0.0050$) and DSO ($r=0.6876$; $P<0.0194$) and inversely correlated with mean apoptotic rate ($r=-0.6791$; $P<0.0216$). Plasma inhibin concentration was correlated with total PAR WT ($r=0.7753$; $P<0.0084$), DSPH ($r=0.7528$; $P<0.0120$) and DSO ($r=0.6863$; $P<0.0197$) and inversely correlated with mean apoptotic rate ($r=-0.6629$; $P<0.0262$). Plasma inhibin has been identified as a useful indicator of stallion reproductive activity (Nagata et al., 1998a). The results of our research indicate that plasma inhibin concentration is more useful as an indicator of stallion fertility than plasma testosterone concentration.

*In vitro* culture techniques were utilized to determine if poor semen quality stallions suffer from receptor-mediated or non-receptor mediated inhibition. HCG treatment was employed to test the receptor-mediated pathway. Testosterone output was not statistically different between poor semen quality and normal stallions following hCG treatment *in vitro* due to a high standard deviation but numerical differences were

present. Forskolin treatment was utilized to test the non-receptor mediated pathway of testosterone production. Testosterone response to forskolin treatment *in vitro* had a tendency to be lower in poor semen quality stallions. Future investigation is needed to identify if results were due to small number of animals. Testis content of StAR protein after hCG treatment did not differ between poor semen quality and normal stallions. Previous reports had indicated a change in StAR mRNA with hCG treatment (Kerban et al., 1999). We were unable to detect changes in StAR protein expression with hCG treatment in the testis. Also, basal expression of StAR and P450scc proteins were not different between normal and poor semen quality stallions. This indicates that StAR transport of cholesterol and P450scc cleavage of cholesterol to pregnenolone do not result in stallion poor semen quality in the animals studied.

These data provide information useful in future research for studying poor semen quality in stallions. Future investigation is needed in order to identify causes of poor semen quality as well as select testicular or hormonal parameters useful in diagnosing poor semen quality stallions.

# CHAPTER VI

## SUMMARY AND CONCLUSIONS

This project was conducted to 1) add important endocrine genes to the horse chromosome map, 2) determine gene expression patterns in the testis of normal and poor semen quality stallions and 3) determine hormonal and seminal parameter changes associated with fertility. The animals in this research study consisted of a normal group and poor semen quality group of stallions. One flaw in this research project was that experimental design was not optimal. The stallions used were not all castrated in the same breeding season. Plus, the stallions were not all the same breed, which means that genetics could have affected semen quality. The stallions in the poor semen quality group of animals were considered to have had poor semen quality for a variety of biological reasons. The poor semen quality animals were not a uniform group, which created a large standard deviation in experimental results.

It is difficult to classify stallions into groups predicted to be either: a) fertile, b) subfertile or c) infertile when one attempts to compare data from different investigators. There appear to be hormonal differences in these three categories of stallions that would be useful as indicators of fertility. More consistent criteria of fertility are needed in order to define these groups for future research.

The following parameters differed between normal and poor semen quality stallions: LT PAR WT, RT PAR WT, total PAR WT, LT DSP/g, RT DSP/g, mean DSP/g, LT DSPT, RT DSPT, DSPH, DSO, motility, PMS, MNS, LT apoptotic rate, RT apoptotic rate and mean apoptotic rate. Apoptotic rate was inversely correlated with all PAR WT measurements and DSP parameters. This information suggests that the amount

of apoptosis or cell death that takes place in the stallion testis is directly related to the size of the testes. Because of this, scrotal width measurements taken when performing BSE evaluations on stallions can be a useful tool in predicting apoptotic rate, DSP, DSO, motility, PMS and MNS. In order for this to be effective, future studies need to be performed to determine the relationship between scrotal width and parenchymal weight.

Many important genes in the stallion testis were evaluated in this study by gene expression. StAR protein was localized in Leydig cells in the interstitial space of the stallion testis by *in situ* hybridization (ISH), as expected from work in other species. StAR protein is important for cholesterol transport from the outer mitochondrial membrane to the inner mitochondrial membrane for steroid biosynthesis (Pollack et al., 1997). The amount of StAR protein mRNA and StAR protein protein in the testis did not differ between normal and poor semen quality stallions. Also, StAR protein expression in the testis in response to hCG stimulation *in vitro* did not differ between normal and poor semen quality stallions. P450scc protein expression in the testis was not different between normal and poor semen quality stallions. The content of StAR protein was correlated with P450scc protein in the testis among all stallions ($r=0.6217$; $P<0.00233$).

Gonadotropins and androgens as well as their receptors in the testis are known to be important factors that can impact male fertility. Circulating testosterone concentrations are readily available through RIA methodology as a diagnostic tool, however plasma testosterone concentration did not differ between normal and poor semen quality stallions. Also, testosterone output in the testis with hCG treatment *in vitro* did not differ between normal and poor semen quality stallions. Testosterone response of parenchyma in cultures to forskolin treatment tended ($P<0.10$) to be lower in

poor semen quality stallions than normal stallions. Motton and Roser (1997) reported no difference in testosterone concentration in poor semen quality stallions compared to normal stallions, however they had a decrease in testosterone concentration in infertile stallions. These data indicate that circulating testosterone concentration is adequate in poor semen quality stallions. Likewise, in our experiments, plasma LH concentration and LHR mRNA expression did not differ between normal and poor semen quality stallions. Plasma FSH tended ($P<0.10$) to be higher in poor semen quality stallions than normal stallions, but FSHR mRNA expression did not differ between the two groups. First, Roser's lab reported an increase in LH and FSH concentration in subfertile stallions (Roser and Hughes, 1992) and secondly, no difference in LH and FSH concentrations in subfertile animals compared to fertile, but a decrease in LH and FSH in infertile stallions (Motton and Roser, 1997). Further investigation is needed to determine if there are changes in LH and FSH concentrations in poor semen quality stallions.

Androgens have also been identified as important hormones for testicular function. In our study, AR was detected by ISH and slot blot mRNA techniques in the stallion testis. AR mRNA expression in the testis did not differ between normal and poor semen quality stallions. Testosterone concentration was inversely correlated with AR mRNA expression in the testis ($r=-0.7907$; $P<0.0022$).

In recent years, estrogen has also been identified in the testis and is thought to play an important role in testis function. In our study, ER alpha mRNA expression tended ($P<0.10$) to be higher in poor semen quality stallions than normal stallions, while ER beta mRNA expression was decreased in poor semen quality stallions. Plasma estradiol concentration was lower in poor semen quality stallions than normal stallions.

Plasma estradiol was correlated with plasma inhibin concentration ($r=0.9391$; $P<0.0001$) as well as parenchymal weight, DSP, DSO, motility, PMS and MNS among all stallions (correlation values stated previously). ER alpha mRNA expression was inversely correlated with ER beta mRNA expression among all stallions ($r=-0.6162$; $P<0.0249$). While ER alpha knockout mice are completely infertile, ER beta knockout mice appear to have no compromised fertility (Couse et al., 2001). This could indicate that the presence of estrogen in the stallion testis is crucial for fertility, but the varying importance of the two isoforms remains unknown. Further studies are necessary in order to elucidate the different roles of ER alpha and ER beta in the stallion testis.

Since glucocorticoids are sometimes used for therapeutic treatment of stallions, our research included the GR as an important gene to study in the testis. In our research, GR alpha, GR beta and GR exon 2 were detected by RPA, ISH and slot blot mRNA techniques in stallion testis. ISH for AR, GR alpha, GR beta and GR exon 2 will need to be repeated in the future to identify localization of these mRNAs in various cell types of the testis. GR alpha and GR exon 2 mRNA expression did not differ between normal and poor semen quality stallions. ISH and mRNA results demonstrate the presence of both GR alpha and GR beta isoforms in the stallion, as observed in the human and rat testes but not the mouse where GR beta is absent. The presence of GR beta in the stallion was unknown previously since the synteny of GR beta is not conserved across species and is absent in the mouse (Otto et al., 1997). The quantity of GR beta mRNA in stallion testes tended to be lower in poor semen quality stallions. Further studies are necessary to determine the physiological significance of GR beta in the stallion testis and the functional role of GR beta in spermatogenesis and steroidogenesis.

Somatogenic/lactogenic hormones have also been identified in the testis and their role in testicular function is still under investigation. In our study, IGF-I and IGF-II mRNA expression did not differ between normal and poor semen quality stallions. The expression of PRLR, GHR and IGF-IR mRNAs were all lower in poor semen quality stallions than normal stallions. Plasma IGF-I tended to be lower in poor semen quality stallions than normal stallions. Plasma IGF-I was correlated with total parenchymal weight ($r=0.06111$; $P<0.0348$), DSPH ($r=0.6772$; $P<0.0156$) and MNS ($r=0.5776$; $P<0.0492$). Hormones such as prolactin, growth hormone and IGF-I appear to play an important autocrine/paracrine role in the testis (Welsh et al., 1986; Spiteri-Grech and Nieschlag, 1993; Gnessi et al., 1997; Schlatt et al., 1997). IGF-I may be a local modulator of testicular function in the stallion since IGF-I has been shown to stimulate the proliferation of Sertoli cells and spermatogonia through the IGF-IR (Jaillard et al., 1987; Soder et al., 1992). Also, GHR knockout mice were shown to have reduced fertility (Chandrashekar et al., 1999) and similarly poor semen quality stallions in this study had a reduced GHR mRNA expression. Further investigation is needed in the stallion to determine the affects of PRLR, GHR and IGF-IR expression on stallion fertility.

Other investigators have indicated inhibin to be a good indicator of stallion fertility (Roser, 2001). We included inhibin in our study to determine if the hormone would be a good indicator of fertility as well as infertility. In our study, βB inhibin mRNA expression and plasma ir-inhibin concentration was lower ($P<0.05$) in poor semen quality stallions than normal stallions. Plasma ir-inhibin was correlated with parenchymal weight, DSP, DSO, motility, PMS and MNS among all stallions (correlation

values stated previously). Kolb et al. (2000) and Yalti et al. (2002) reported inhibin concentration was correlated with sperm production in the human testis. The same results were found in the stallions in this study. Plasma inhibin concentrations have been reported to be lower in elderly men (Mahmoud et al., 2003). Plasma ir-inhibin concentration was also reported to be lower in poor semen quality stallions than normal stallions (Stewart and Roser, 1998). Since inhibin is an important regulator of gonadotroph secretion and paracrine regulation of testicular function (Marchetti et al., 2003), changes in inhibin secretion might be an indicator of fertility status (von Eckardstein et al., 1999; Hu et al., 2003). Plasma inhibin results indicate that inhibin concentration is a useful and non-invasive method for the evaluation of male subfertility and is correlated with sperm production (Stewart and Roser, 1997; Yalti et al., 2002). Further investigation is needed to determine if plasma inhibin concentration can be used as a tool for identifying male fertility status.

We have now placed two important endocrine genes on the horse cytogenetic map. *LHR* maps to the same chromosome that contains follicle-stimulating hormone receptor (*FSHR*) (Chowdhary et al., 2002). Equine glucocorticoid receptor mapped to chromosome 14q16-q21, a gene poor region in need of further cytogenetic mapping data. Equine luteinizing hormone receptor mapped to chromosome 15q22-q23. By identification of the chromosomal location of *GR* we have also added information to a gene poor region of the ECA 14 map. Equine specific probes are now available for clarification of the role of *LHR* in reproduction, and primers that amplify the *GR* isoforms are available for use in gene expression studies of glucocorticoid resistance. The chromosomal locations of *LHR* and *GR* are now available for further study of infertility

or genetic diseases associated with glucocorticoids. Polymorphisms in and around these genes could be studied in horse families to determine whether there are associations between these genes and infertility.

This project was conducted to 1) add important endocrine genes to the horse chromosome map, 2) determine gene expression patterns in the testis of normal and poor semen quality stallions (Tables 4 and 5) and 3) determine hormonal and seminal parameter changes associated with semen quality. As a result of this research, two important endocrine genes were added to the horse chromosome map. StAR protein was localized to Leydig cells in the interstitial space of the stallion testis. The AR and both isoforms of GR were detected in the stallion testis. The presence of GR beta was proven and was unknown previously in the stallion. The expression of PRLR, GHR and IGF-IR mRNAs were all lower in the testis of poor semen quality stallions compared to normal stallions. ER beta mRNA expression and plasma estradiol concentration were significantly lower in poor semen quality stallions. βB inhibin mRNA expression and plasma ir-inhibin concentration was lower in poor semen quality stallions than normal stallions. This supports the concept that plasma inhibin concentration is one tool for determining male fertility status. These data provide preliminary information and tools for future research to determine the gene expression and hormonal changes associated with stallion poor semen quality.

Table 4. Gene expression results that did not differ between normal and poor semen quality stallions.

| mRNA | Normal Stallion Mean (density of pixels) | Normal Stallion Standard Deviation (density of pixels) | Poor Semen Quality Stallion Mean (density of pixels) | Poor Semen Quality Stallion Standard Deviation (density of pixels) | P-value |
|---|---|---|---|---|---|
| **FSHR** | 2.70x10e6 | 1.15x10e5 | 2.62x10e6 | 1.55x10e5 | 0.72 |
| **LHR** | 2.52x10e6 | 1.49x10e5 | 2.38x10e6 | 2.01x10e5 | 0.64 |
| **StAR** | 2.78x10e7 | 2.09x10e6 | 2.99x10e7 | 2.82x10e6 | 0.61 |
| **GR alpha** | 1.09x10e6 | 3.12x10e4 | 1.12x10e6 | 4.21x10e4 | 0.69 |
| **GR exon 2** | 4.29x10e6 | 1.48x10e5 | 4.15x10e6 | 2.00x10e5 | 0.63 |
| **AR** | 3.75x10e6 | 7.88x10e4 | 3.77x10e6 | 1.06x10e5 | 0.91 |
| **IGF-I** | 2.00x10e6 | 6.87x10e4 | 2.00x10e6 | 9.27x10e4 | 0.99 |
| **IGF-II** | 3.56x10e7 | 1.40x10e6 | 3.71x10e7 | 1.90x10e6 | 0.58 |
| **ER alpha** | 2.71x10e6 | 6.54x10e4 | 2.93x10e6 | 8.83x10e4 | 0.09 |
| **GR beta** | 8.64x10e5 | 1.99x10e4 | 7.89x10e5 | 2.69x10e4 | 0.07 |

Table 5. Gene expression results that differed between normal and poor semen quality stallions.

| mRNA | Normal Stallion Mean (density of pixels) | Normal Stallion Standard Deviation (density of pixels) | Poor Semen Quality Stallion Mean (density of pixels) | Poor Semen Quality Stallion Standard Deviation (density of pixels) | P-value |
|---|---|---|---|---|---|
| **ER beta** | 1.84x10e7 | 7.53x10e5 | 1.39x10e7 | 1.02x10e6 | 0.01 |
| **βB inhibin** | 2.61x10e7 | 9.86x10e5 | 2.18x10e7 | 1.33x10e6 | 0.04 |
| **PRLR** | 6.73x10e7 | 1.57x10e6 | 5.80x10e7 | 2.12x10e6 | 0.01 |
| **GHR** | 2.24x10e6 | 8.11x10e4 | 1.72x10e6 | 1.09x10e5 | 0.01 |
| **IGF-IR** | 7.14x10e5 | 2.27x10e4 | 5.87x10e5 | 3.06x10e4 | 0.01 |

## LITERATURE CITED

Amann, R. P. 1962. Reproductive capacity of dairy bulls. IV. Spermatogenesis and testicular germ cell degeneration. Am. J. Anat. 110:69-78.

Ascoli, M., F. Fanelli, and D. L. Segaloff. 2002. The lutropin/choriogonadotropin receptor, a 2002 perspective. Endo. Rev. 23:141-174.

Aspden, W. J., R. J. Rodgers, D. M. Stocco, P. T. Scott, N. G. Wreford, T. E. Trigg, J. Walsh, and M. J. D'Occhio. 1998. Changes in testicular steroidogenic acute regulatory (STAR) protein, steroidogenic enzymes and testicular morphology associated with increased testosterone secretion in bulls receiving the luteinizing hormone releasing hormone agonist deslorelin. Domest. Anim. Endocrinol. 15:227-238.

Baccetti, B., G. Collodel, E. Costantino-Ceccarini, A. Eshkol, L. Gambera, E. Moretti, M. Strazza, and P. Piomboni. 1998. Localization of human follicle-stimulating hormone in the testis. Faseb J. 12:1045-54.

Bielinska, M., H. Parviainen, S. B. Porter-Tinge, S. Kiiveri, and E. Genova. 2003. Adrenocortical tumor formation correlates with the expression of GATA-4 and luteinizing hormone receptor. Endocrinology 144:4123-4133.

Bilby, C. R., J. F. Bader, B. E. Salfen, R. S. Youngquist, C. N. Murphy, H. A. Garverick, B. A. Crooker, and M. C. Lucy. 1999. Plasma GH, IGF-I, and conception rate in cattle treated with low doses of recombinant bovine GH. Therio. 51:1285-1296.

Binart, N., N. Melaine, C. Pineau, H. Kercret, A. M. Touzalin, P. Imbert-Bollore, P. A. Kelly, and B. Jegou. 2003. Male reproductive function is not affected in prolactin receptor-deficient mice. Endocrinology 144:3779-3782.

Bonne, A. C., M. G. den Bieman, G. F. Gillissen, H. A. van Lith, and L. F. van Zutphen. 2002. Chromosomal localization of genes involved in biosynthesis, metabolism or transport of cholesterol in the rat. Genome Res. 97:183-186.

Cailleau, J., S. Vermeire, and G. Verhoeven. 1990. Independent control of the production of insulin-like growth factor I and its binding protein by cultured testicular cells. Mol. Cell Endocrinol. 69:79-89.

Chandrashekar, V., A. Bartke, K. T. Coschigano, and J. J. Kopchick. 1999. Pituitary and testicular function in growth hormone receptor gene knockout mice. Endocrinology 140:1082-1088.

Chandrashekar, V., A. Bartke, C. A. Awoniyi, C. H. Tsai-Morris, M. L. Dufau, L. D. Russell, and J. J. Kopchick. 2001. Testicular endocrine function in GH receptor gene disrupted mice. Endocrinology 142:3443-3450.

Chowdhary, B. P., T. Raudsepp, D. Honeycutt, E. K. Owens, F. Piumi, G. Guerin, T. C. Matise, S. R. Kata, J. E. Womack, and L. C. Skow. 2002. Construction of a 5000(rad) whole-genome radiation hybrid panel in the horse and generation of a comprehensive and comparative map for ECA11. Mamm. Genome 13:89-94.

Chowdhary, B. P., T. Raudsepp, S. R. Kata, G. Goh, L. V. Millon, V. Allan, F. Piumi, G. Guerin, J. Swinburne, M. Binns, T. L. Lear, J. Mickelson, J. Murray, D. F. Antczak, J. E. Womack, and L. C. Skow. 2003. The first-generation whole-genome radiation hybrid map in the horse identifies conserved segments in human and mouse genomes. Genome Res. 13:742-51.

Clermont, Y. 1962. Quantitative analysis of spermatogenesis of the rat: a revised model for the renewal of spermatogonia. Am. J. Anat. 111:111-129.

Couse, J. E., D. Mahato, E. M. Eddy, and K. S. Korach. 2001. Molecular mechanism of estrogen action in the male: insights from the estrogen receptor null mice. Reprod. Fertil. Dev. 13:211-219.

De Gendt, K., J. V. Swinnen, P. T. Saunders, L. Schoonjans, M. Dewerchin, A. Devos, K. Tan, N. Atanassova, F. Claessens, C. Lecureuil, W. Heyns, P. Carmeliet, F. Guillou, R. M. Sharpe, and G. Verhoeven. 2004. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc. Natl. Acad. Sci. USA 101:1327-1332.

De Kretser, D. M., K. J. Catt, and C. A. Paulsen. 1971. Studies on the *in vitro* testicular binding of iodinated luteinizing hormone in rats. Endocrinology 88:332-337.

De Kretser, D. M., G. P. Risbridger, and J. B. Kerr. 1995. Basic endocrinology of the testis, pp.2307-2335. In: Endocrinology, L. J. DeGroot ed., W. B. Saunders Company, Philadelphia, Pennsylvania.

Denger, S., G. Reid, H. Brand, M. Kos, and F. Gannon. 2001. Tissue-specific expression of human ERalpha and ERbeta in the male. Mol. Cell Endocrinol. 178:155-160.

Dohle, G. R., M. Smit, and R. F. Weber. 2003. Androgens and male fertility. World J. Urol. 21:341-345.

Epstein, L. F. and N. R. Orme-Johnson. 1991. Regulation of steroid hormone biosynthesis: identification of precursors of a phosphoprotein targeted to the mitochondrion in stimulated rat adrenal cortex cells. J. Biol. Chem. 266:19739-19745.

Feelders, R. A., S. W. Lamberts, L. J. Hofland, P. M. van Koetsveld, M. Verhoef-Post, A. P. Themmen, F. H. de Jong, H. J. Bonjer, A. J. Clark, A. J. van der Lely, and W. W. de Herder. 2003. Luteinizing hormone (LH)-responsive Cushing's syndrome: the demonstration of LH receptor messenger ribonucleic acid in hyperplastic adrenal cells, which respond to chorionic gonadotropin and serotonin agonists *in vitro*. J. Clinical Endo. and Metab. 88:230-237.

Francke, U. and U. Gehring. 1980. Chromosome assignment of a murine glucocorticoid receptor gene (Grl-1) using intraspecies somatic cell hybrids. Cell 22:657-664.

French, J. T. 1988. In vitro regulation of procine Leydig cell steroidogenesis by insulin-like growth factor I (IGF-I) and the phorbol ester 12-myristate-13-acetate (PMA) (M.S. Thesis, Texas A&M University, College Station, TX).

French J. T. and T. H. Welsh, Jr. 1990. *In vitro* modulation of porcine Leydig cell steroidogenesis by phorbol-12-myristate-13-acetate and 1,2-dioctanoylglycerol. Acta Endocrinol. 122:101-106.

Fujimura, S., E. Hondo, T. Kobayashi, K. Yamanouchi, N. Inoue, S. Nagata, G. Watanabe, K. Taya, N. Kitamura, and J. Yamada. 1998. Expression of inhibin alpha-subunit in horse testis. J. Vet. Med. Sci. 60:937-942.

Garmey, J. C., H. D. Guthrie, W. M. Garrett, M. H. Stoler, and J. D. Veldhuis. 2000. Localization and expression of low-density lipoprotein receptor, steroidogenic acute regulatory protein, cytochrome P450 side-chain cleavage and P450 17-alpha-hydroxylase/C17-20 lyase in developing swine follicles: *in situ* molecular hybridization and immunocytochemical studies. Mol. Cell Endocrinol. 170:57-65.

Gehring, U., B. Segnitz, B. Foellmer, and U. Francke. 1985. Assignment of the human gene for the glucocorticoid receptor to chromosome 5. Proc. of the Natl Acad. of Sciences of the USA 82:3751-3755.

Gnessi, L., A. Fabbri, and G. Spera. 1997. Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and local environment. Endo. Rev. 18:541-609.

Godfrey, R. W., D. D. Lunstra, T. G. Jenkins, J. G. Berardinelli, M. J. Guthrie, D. A. Neuendorff, C. R. Long, and R. D. Randel. 1990. Effect of season and location on semen quality and serum concentrations of luteinizing hormone and testosterone in Brahman and Hereford bulls. J. Anim. Sci. 68:734-749.

Goldner-Sauve, A., C. Szpirer, J. Szpirer, G. Levan, and D. L. Gasser. 1991. Chromosome assignments of the genes for glucocorticoid receptor, myelin basic protein, leukocyte common antigen, and TRPM2 in the rat. Biochem. Genetics 29:275-286.

Guillaumot, P. and M. Benahmed. 1999. Prolactin receptors are expressed and hormonally regulated in rat Sertoli cells. Mol. Cell Endocrinol. 149:163-168.

Heninger, N. L., C. Staub, T. L. Blanchard, L. Johnson, D. D. Varner, and D. W. Forrest. 2004. Germ cell apoptosis in the testes of normal stallions. Therio. 62:283-297.

Hess, M. F. and J. F. Roser. 2001. The effects of age, season and fertility status on plasma and intratesticular insulin-like growth factor I concentration in stallions. Therio. 56:723-733.

Hondo, E., M. Kurohmaru, S. Sakai, K. Ogawa, and Y. Hayashi. 1995. Prolactin receptor expression in rat spermatogenic cells. Biol. Reprod. 52:1284-1290.

Hu, Y. A., Y. F. Huang, J. P. Xu, and P. Y. Zhu. 2003. Inhibin B levels of serum and seminal plasma in fertile and infertile males. Zhonghua Nan Ke Xue 9:447-450.

Huckins, C. 1978. The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium. Anat. Rec. 190:905-926.

Irvine, C. H. and S. L. Alexander. 1991. Effect of sexual arousal on gonadotrophin-releasing hormone, luteinizing hormone and follicle-stimulating hormone secretion in the stallion. J. Reprod. Fertil. 44:135-143.

Jabbour, H. N. and G. A. Lincoln. 1999. Prolactin receptor expression in the testis of the ram: localization, functional activation and the influence of gonadotrophins. Mol. Cell Endocrinol. 148:151-161.

Jaillard, C., P. G. Chatelain, and J. M. Saez. 1987. *In vitro* regulation of pig Sertoli cell growth and function effects of fibroblast growth factor and somatomedin-C. Biol. Reprod. 37:665-674.

Johnson, L., G. K. Carter, D. D. Varner, T. S. Taylor, T. L. Blanchard, and M. S. Rembert. 1994. The relationship of daily sperm production with number of Sertoli cells and testicular size in adult horses: role of primitive spermatogonia. J. Reprod. Fertil. 100:315-321.

Juhasz, J., P. Nagy, M. Kulcsar, and G. Y. Huszenicza. 2000. Methods for semen and endocrinological evaluation of the stallion: a review. Acta Vet. Brno 69: 247-259.

Kaipia, A., T. L. Penttila, and J. Toppari. 1994. Follicle-stimulating hormone regulation of inhibin alpha-subunit mRNA in staged rat seminiferous tubules. Eur. J. Endocrinol. 131:323-329.

Kerban, A., D. Boerboom, and J. Sirois. 1999. Human chorionic gonadotripin induces an inverse regulation of steroidogenic acute regulatory protein messenger ribonucleic acid in theca interna and granulosa cells of equine preovulatory follicles. Endocrinology 140:667-674.

King, S. R. T. Ronen-Fuhrmann, R. Timberg, B. J. Clark, J. Orly, and D. M. Stocco. 1995. Steroid production after *in vitro* transcription, translation, and mitochondrial processing of protein products of complementary deoxyribonucleic acid for steroidogenic acute regulatory protein. Endocrinology 136:5165-5176.

Klaij, I. A., M. A. Timmerman, L. J. Blok, J. A. Grootegoed, and F. H. de Jong. 1992. Regulation of inhibin beta B-subunit mRNA expression in rat Sertoli cells: consequences for the production of bioactive and immunoreactive inhibin. Mol. Cell Endocrinol. 85:237-246.

Klaij, I. A., A. M. van Pelt, M. A. Timmerman, L. J. Blok, D. G. de Rooij, and F. H. de Jong. 1994. Expression of inhibin subunit mRNAs and inhibin levels in the testis of rats with stage-synchronized spermatogenesis. J. Endocrinol. 141:131-141.

Knudson, C. M., K. S. Tung, W. G. Tourtellotte, G. A. Brown, and S. J. Korsmeyer. 1995. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270:96-99.

Koike, S. and T. Noumura. 1995. Immunohistochemical localization of insulin-like growth factor-II in the perinatal rat gonad. Growth Regul. 5:185-189.

Kolb, B. A., F. Z. Stanczyk, and R. Z. Sokol. 2000. Serum inhibin B levels in males with gonadal dysfunction. Fertil. Steril. 74:234-238.

Kotula, M., R. Tuz, B. Fr czek, A. Wojtusiak, and B. Bilinska. 2000. Immunolocalization of androgen receptors in testicular cells of prepubertal and pubertal pigs. Folia Histochem. Cytobiol. 38:157-162.

Kotula, M., E. Koziel, J. Sadowska, M. Gancarczyk, and B. Bilinska. 2001. Immunofluorescent localization of the StAR protein in mitochondria of mouse Leydig cells *in vitro*. Folia Histochem. Cytobiol. 39:169-170.

Leers-Sucheta, S., D. M. Stocco, and S. Azhar. 1999. Down-regulation of steroidogenic acute regulatory (StAR) protein in rat Leydig cells: implications for regulation of testosterone production during aging. Mech. Ageing Dev. 107:197-203.

Lobie, P. E., W. Breipohl, J. G. Aragon, and M. J. Waters. 1990. Cellular localization of the growth hormone receptor/binding protein in the male and female reproductive systems. Endocrinology 126:2214-2221.

MacAdams, M. R., R. H. White, and B. E. Chipps. 1986. Reduction of serum testosterone levels during chronic glucocorticoid therapy. Annals of Internal Medicine 104:648-651.

Mahmoud, A. M., S. Goemaere, Y. El-Garem, I. Van Pottelbergh, F. H. Comhaire, and J. M. Kaufman. 2003. Testicular volume in relation to hormonal indices of gonadal function in community-dwelling elderly men. J. Clin. Endocrinol. Metab. 88:179-184.

Makinen, S., S. Makela, Z. Weihua, M. Warner, B. Rosenlund, S. Salmi, O. Hovatta, and J. K. Gustafsson. 2001. Localization of oestrogen receptors alpha and beta in human testis. Mol. Hum. Reprod. 7:497-503.

Marchetti, C., M. Hamdane, V. Mitchell, K. Mayo, L. Devisme, J. M. Rigot, J. C. Beauvillain, E. Hermand, and A. Defossez. 2003. Immunolocalization of inhibin and activin alpha and betaB subunits and expression of corresponding messenger RNAs in the human adult testis. Biol. Reprod. 68:230-235.

McKay, L. A. and J. A. Cidlowski. 1999. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kB and steroid receptor-signaling pathways. Endo. Rev. 20:435-459.

McKenna, T. J., D. Lorber, A. Lacroix, and D. Rabin. 1979. Testicular activity in Cushing's disease. Acta Endocrinol. 91:501-510.

Means, A. R. and J. Vaitukaitis. 1972. Peptide hormone receptors: specific binding of $^{131}$I-FSH to testis. Endocrinology 90:39-46.

Mikola, M., J. Kero, J. H. Nilson, R. A. Keri, M. Poutanen, and I. Huhtaniemi. 2003. High levels of luteinizing hormone analog stimulate gonadal and adrenal tumorigenesis in mice transgenic for the mouse inhibin-alpha-subunit promoter/Simian virus 40 T-antigen fusion gene. Oncogene 22:3269-3278.

Moore, A. and I. D. Morris. 1993. The involvement of insulin-like growth factor-I in local control of steroidogenesis and DNA synthesis of Leydig and non-Leydig cells in the rat testicular interstitium. J. Endocrinol. 138:107-114.

Motton, D. D. and J. F. Roser. 1997. HCG binding to the testicular LH receptor is similar in fertile, subfertile and infertile stallions. J. Androl. 18:411-416.

Mowa, C. N. and T. Iwanaga. 2001. Expression of estrogen receptor-alpha and –beta mRNAs in the male reproductive system of the rat as revealed by *in situ* hybridization. J. Mol. Endocrinol. 26:165-174.

Mueller, S. O. and K. S. Korach. 2001. Immortalized testis cell lines from estrogen receptor (ER) alpha knock-out and wild-type mice expressing functional ERalpha or ERbeta. J. Androl. 22:652-664.

Mulder, E., M. J. Peters, J. De Vries, and H. J. van der Molen. 1975. Characterization of a nuclear receptor for testosterone in seminiferous tubules of mature rat testes. Mol Cell Endocrinol. 2:171-182.

Muyan, M., J. F. Roser, N. Dybdal, and D. M. Baldwin. 1993. Modulation of gonadotropin-releasing hormone-stimulated luteinizing hormone release in cultured male equine anterior pituitary cells by gonadal steroids. Biol. Reprod. 49:340-345.

Nagata, S., Y. I. Miyake, Y. Nambo, N. Nagamine, G. Watanabe, N. Tsunoda, H. Taniyama, E. Hondo, J. Yamada, and K. Taya. 1998a. Inhibin secretion in the stallion. Equine Vet. J. 300:98-103.

Nagata, S., N. Tsunoda, N. Nagamine, Y. Tanaka, H. Taniyama, Y. Nambo, G. Watanabe, and K. Taya. 1998b. Testicular inhibin in the stallion: cellular source and seasonal changes in its secretion. Biol. Reprod. 59:62-68.

Namiki, M., K. Yokokawa, A. Okuyama, E. Koh, H. Kiyohara, M. Nakao, S. Sakoda, K. Matsumoto, and T. Sonoda. 1991. Evidence for the presence of androgen receptors in human Leydig cells. J. Steroid Biochem. Mol. Biol. 38:79-82.

Orth, J. and A. K. Christensen. 1977. Localization of $^{125}$I-labelled FSH in the testes of hypophysectomized rats by autoradiography at the light and electron microscopic levels. Endocrinology 101:262-278.

Orth, J. and A. K. Christensen. 1978. Autoradiographic localization of specifically bound 125I-labelled follicle stimulating hormone on spermatogonia of the rat testis. Endocrinology 103:1944-1951.

Otto, C., H. M. Reichardt, and G. Schutz. 1997. Absence of glucocorticoid receptor-beta in mice. J. Biol. Chemistry 272:26665-26668.

Pelletier, G., C. Labrie, and F. Labrie. 2000. Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs. J. Endocrinology 165:359-370.

Pinaud, M. A., J. F. Roser, and N. Dybdal. 1991. Gonadotropin releasing hormone (GnRH) induced luteinizing hormone (LH) secretion from perifused equine pituitaries. Domest. Anim. Endo. 8:353-368.

Pollack, S. E., E. E. Furth, C. B. Kallen, F. Arakane, M. Kiriakidou, K. F. Kozarsky, and J. F. Strauss III. 1997. Localization of the steroidogenic acute regulatory protein in human tissues. J. Clinical Endocrinol. and Metab. 82:4243-4251.

Pujols, L., J. Mullol, J. Roca-Ferrer, A. Torrego, A. Xaubet, J. A. Cidlowski, and C. Picado. 2002. Expression of glucocorticoid receptor alpha- and beta-isoforms in human cells and tissues. Am. J. Phys. Cell Phys. 283:C1324-1331.

Rodriguez, I., C. Ody, K. Araki, I. Garcia, and P. Vassalli. 1997. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. EMBO J. 16:2262-2270.

Roser, J. F. 2001. Endocrine and paracrine control of sperm production in stallions. Anim. Reprod. Sci. 68:139-151.

Roser, J. F. and J. P. Hughes. 1991. Prolonged pulsatile administration of gonadotrophin-releasing hormone (GnRH) to fertile stallions. J Reprod. Fertil. 44:155-168.

Roser, J. F. and J. P. Hughes. 1992. Dose-response effects of gonadotropin-releasing hormone on plasma concentrations of gonadotropins and testosterone in fertile and subfertile stallions. J. Androl. 13:543-550.

Roser, J. F., P. M. McCue, and E. Hoye. 1994. Inhibin activity in the mare and stallion. Domest. Anim. Endo. 11:87-100.

Rousseau-Merck, M. F., M. Misahi, M. Atger, H. Loosfelt, E. Milgrom, and R. Berger. 1990. Localization of the human luteinizing hormone/choriogonadotropin receptor gene (LHCGR) to chromosome 2p21. Cyto. and Cellular Genetics 54:77-79.

Rowland, T. L., S. M. McHugh, J. Deighton, R. J. Dearman, P. W. Ewan, and I. Kimber. 1998. Differential regulation by thalidomide and dexamethasone of cytokine expression in human peripheral blood mononuclear cells. Immunopharm. 40:11-20.

Ruizeveld de Winter, J. A., J. Trapman, M. Vermey, E. Mulder, N. D. Zegers, and T. H. der Kwast. 1991. Androgen receptor expression in human tissues: an immunohistochemical study. J. Histochem. Cytochem. 39:927-936.

Russell, L. D., H. Chiarini-Garcia, S. J. Korsmeyer, and C. M. Knudson. 2002. Bax-dependent spermatogonia apoptosis is required for testicular development and spermatogenesis. Biol. Reprod. 66:950-958.

Saez, J. M. 1994. Leydig cells: endocrine, paracrine, and autocrine regulation. Endo. Rev. 15:574-626.

Saint-Dizier, M., M. Chopineau, J. Dupont, P. F. Daels, and Y. Combarnous. 2003. Expression and binding activity of luteinizing hormone/chorionic gonadotropin receptors in the primary corpus luteum during early pregnancy in the mare. Biol. Reprod. 69:1743-1749.

Sanborn, B. M., A. Steinberger, R. K. Tcholakian, and E. Steinberger. 1977. Direct measurement of androgen receptors in cultured Sertoli cells. Steroids 29:493-502.

Schaaf, M. J. and J. A. Cidlowski. 2002. Molecular mechanisms of glucocorticoid action and resistance. J. Steroid Bioch. and Mol. Biology 83:37-48.

Schaaf, M. J. and J. A. Cidlowski. 2003. Molecular determinants of glucocorticoid receptor mobility in living cells: the importance of ligand affinity. Mol. Cell Biology 23:1922-1934.

Schibler, L., D. Vaiman, A. Oustry, N. Guinec, A. L. Dangy-Caye, A. Billault, and E. P. Cribiu. 1998. Construction and extensive characterization of a goat bacterial artificial chromosome library with threefold genome coverage. Mamm. Genome 9:119-124.

Schlatt, S., A. Meinhardt, and E. Nieschlag. 1997. Paracrine regulation of cellular interactions in the testis: factors in search of a function. Eur. J. Endocrinol. 137:107-117.

Schmidt, M., H. G. Pauels, N. Lugering, A. Lugering, W. Domschke, and T. Kucharzik. 1999. Glucocorticoids induce apoptosis in human monocytes: potential role of IL-1 beta. J. Immunology 163:3484-3490.

Seamon, K. B. and J. W. Daly. 1981. Forskolin: a unique diterpene activator of cyclic AMP generating systems. J. Cyclic Nucleotide Res. 24:201.

Senger, P. L. 1999. Male endocrinology and spermatogenesis, pp. 168-186. In: Pathways to Pregnancy and Parturition, P. L. Senger ed., The Mack Printing Group-Science Press, Ephrata, Pennsylvania.

Shughrue, P. J., M. V. Lane, P. J. Scrimo, and I. Merchenthaler. 1998. Comparative distribution of estrogen receptor-alpha (ER-alpha) and beta (ER-beta) mRNA in the rat pituitary, gonad, and reproductive tract. Steroids 63:498-504.

Soder, O., P. Bang, A. Wahab, and M. Parvinen. 1992. Insulin-like growth factors selectively stimulate spermatogonial, but not meiotic, deoxyribonucleic acid synthesis during rat spermatogenesis. Endocrinology 131:2344-2350.

Sonstegard, T. S., W. M. Garrett, M. S. Ashwell, G. L. Bennett, S. M. Kappes, and C. P. Tassell. 2000. Comparative map alignment of BTA27 and HSA4 and 8 to identify conserved segments of genome containing fat deposition QTL. Mamm. Genome 11:682-688.

Spiteri-Grech, J. and E. Nieschlag. 1993. Paracrine factors relevant to the regulation of spermatogenesis: a review. J. Reprod. Fertil. 98:1-14.

Stewart, B.L. and J.F. Roser. 1998. Effects of age, season, and fertility status on plasma and intratesticular immunoreactive (IR) inhibin concentrations in stallions. Dom. Anim. Endo. 15:129-139.

Stocco, D. M. and T. C. Sodeman. 1991. The 30-kDa mitochondrial proteins induced by hormone stimulation in MA-10 mouse Leydig tumor cells are processed from larger precursors. J. Biol. Chem. 266:19731-19738.

Stocco, D. M. and B. J. Clark. 1996. Regulation of the acute production of steroids in steroidogenic cells. Endo. Rev. 17:221-243.

Sugawara, T., J. A. Holt, D. Driscoll, J. F. Strauss 3rd, D. Lin, W. L. Miller, D. Patterson, K. P. Clancy, I. M. Hart, and B. J. Clark. 1995. Human steroidogenic acute regulatory protein: functional activity in COS-1 cells, tissue-specific expression, and mapping of the structural gene to 8p11.2 and a pseudogene to chromosome 13. Proc. Natl. Acad. Sci. USA 92:4778-4782.

Sundby, A., D. Andersen, K. Purvis, and V. Hansson. 1984. Testicular gonadotropin receptors, testicular testosterone, dihydrotestosterone and androstenedione in the developing bull. Arch. Androl. 12:59-64.

Thompson, Jr., D. L., B. W. Pickett, W. E. Berndtson, J. L. Voss, and T. M. Mett. 1977. Reproductive physiology of the stallion. VIII. Artificial photoperiod, collection interval and seminal characteristics, sexual behavior and concentrations of LH and testosterone in serum. J. Anim. Sci. 44:656-664.

Thompson, Jr., D. L. and P. G. Honey. 1984. Active immunization of Prepubertal colt against estrogens: hormonal and testicular responses after puberty. J. Anim. Sci. 59:189-196.

Thompson, W. E., J. Powell, K. H. Thomas, and J. A. Whittaker. 1999. Immunolocalization and expression of the steroidogenic acute regulatory protein during the transitional stages of rat follicular differentiation. J. Histochem. Cytochem. 47:769-776.

Vannelli, B. G., T. Barni, C. Orlando, A. Natali, M. Serio, and G. C. Balboni. 1988. Insulin-like growth factor-I (IGF-I) and IGF-I receptor in human testis: an immunohistochemical study. Fertil. Steril. 49:666-669.

Varner, D. D., S. D. Vaughan, and L. Johnson. 1991. Use of a computerized system for evaluation of equine spermatozoal motility. Am. J. Vet. Res. 52:224-230.

Varner, D. D., T. L. Blanchard, S. P. Brinsko, C. C. Love, T. S. Taylor, and L. Johnson. 2000. Techniques for evaluating selected reproductive disorders of stallions. Anim. Reprod. Sci. 60:493-509.

Verhoeven, G. 1980. Androgen receptor in cultured interstitial cells derived from immature rat testis. J. Steroid Biochem. 13:469-473.

von Eckardstein, S., M. Simoni, M. Bergmann, G. F. Weinbauer, P. Gassner, A. G. Schepers, and E. Nieschlag. 1999. Serum inhibin B in combination with serum follicle-stimulating hormone (FSH) is a more sensitive marker than serum FSH alone for impaired spermatogenesis in men, but cannot predict the presence of sperm in testicular tissue samples. J. Clin. Endocrinol. Metab. 84:2496-2501.

Wahlstrom, T., I. Huhtaniemi, O. Hovatta, and M. Seppala. 1983. Localization of luteinizing hormone, follicle-stimulating hormone, prolactin, and their receptors in human and rat testis using immunohistochemistry and radioreceptor assay. J. Clin. Endocrinol. Metab. 57:825-830.

Watson, E. D., S. R. Thomson, and A. F. Howie. 2000. Detection of steroidogenic acute regulatory protein in equine ovaries. J. Repro. Fertility 119:187-192.

Weber, M. A., S. Groos, U. Hopfl, M. Spielmann, G. Aumuller, and L. Konrad. 2000. Glucocorticoid receptor distribution in the rat testis during postnatal development and effects of Dexamethasone on immature peritubular cells *in vitro*. Andrologia 32:23-30.

Welsh, Jr., T. H., R. L. McCraw, and B. H. Johnson. 1979. Influence of corticosteroids on testosterone production in the bull. Biol. Repro. 21:755-763.

Welsh, Jr., T. H., T. H. Bambino, and A. J. W. Hsueh. 1982. Mechanism of glucocorticoid-induced suppression of testicular androgen biosynthesis *in vitro*. Biol. Repro. 27:1138-1146.

Welsh, Jr., T. H., B. G. Kasson, and A. J. Hsueh. 1986. Direct biphasic modulation of gonadotropin-stimulated testicular androgen biosynthesis by prolactin. Biol. Reprod. 34:796-804.

Wilcox, J. 1993. Fundamental principles of *in situ* hybridization. J. Histochemistry and Cytochemistry 41:1725-1733.

Yalti, S., B. Gurbuz and C. Ficicioglu. 2002. Serum levels of inhibin B in men and their relationship with gonadal hormones, testicular volume, testicular biopsy results and sperm parameters. J. Obstet. Gynaecol. 22:649-654.

Yuan, B. Z., Y. Yang, C. L. Keck-Waggoner, D. B. Zimonjic, S. S. Thorgeirsson, and N. C. Popescu. 1999. Assignment and cloning of mouse Arhgap7 to chromosome 8A4-B2, a conserved syntenic region of human chromosome 8p22--->p21. Cytogenet. Cell Genet. 87:189-190.

Yudt, M. R. and J. A. Cidlowski. 2002. The glucocorticoid receptor: coding a diversity of proteins and responses through a single gene. Mol. Endo. 16:1719-1726.

Yudt, M. R., C. M. Jewell, R. J. Bienstock, and J. A. Cidlowski. 2003. Molecular origins for the dominant negative function of human glucocorticoid receptor beta. Mol. Cell Biology 23:4319-4330.

Zhang, F. P., A. Rannikko, J. Toppari, A. Bartke, and I. Huhtaniemi. 1995. Development expression of the prolactin receptor gene in rat gonads. J. Endocrinol. 147:497-505.

Zhou, Q., R. Nie, G. S. Prins, P. T. Saunders, B. S. Katzenellenbogen, and R. A. Hess. 2002. Localization of androgen and estrogen receptors in adult male mouse reproductive tract. J. Androl. 23:870-881.

# APPENDIX A

## FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) PROTOCOL

**Procedures described by Chowdhary et al. (2002, 2003)**

### A. Probe Preparation

1. Label probes with biotin by nick translation (BioNick Labeling System, Invitrogen Corp. cat# 18247-015).
   1 µg probe DNA
   5 µl 10X dNTP mix (T, G, C, bio-dATP)
   5 µl 10X enzyme mix (DNA Polymerase I and DNase I)
   dd water up to 45 µl
2. Mix reaction up and down with pipette and incubate at 16°C for 1 hour and 15 minutes.
3. Purify the labeled probe through a Qiagen QIAquick PCR column (Qiagen, Inc., Valencia, CA).
4. Check the size and quantity of the labeled probe on a 1% agarose gel using 1 µl of labeled probe and 1 µl Orange G dye. Use 100 bp marker (New England Biolabs, Beverly, MA) to identify size.
5. Run the gel at 100 volts for approximately 30 minutes. The labeled probe should yield or result in a smear in the size range of 200 to 600 bp.

### B. Chromosome Slide Preparation

1. Under the microscope choose suitable hybridization area and mark it with a glass pencil on the opposite side of each slide by identifying dense regions of elongated chromosomes under the microscope.
2. Apply 500 µl of RNase working solution to the slide and cover slip the slide.
3. Incubate at 37°C for 1 hour.
4. Wash the slides in 2X SSC for a maximum of 5 minutes.
5. Pass the slides through the following washes:

| | |
|---|---|
| 70% ethanol | 2 minutes |
| 80% ethanol | 2 minutes |
| 90% ethanol | 2 minutes |
| 100% ethanol | 2 minutes |

6. Allow slides to air dry.

### C. Hybridization

1. Make master mix of hybridization mix:
   36 ml 100% formamide
   7 g dextran sulfate
   7.5 ml 20X SSC
   Mix and store in 200 µl aliquots at –20°C. Mix 7:3 master mix to DNA for hybridization.

2. Denature chromosomes in 70% formamide, 2X SSC at 70ºC for 2 minutes and immediately pass through ice cold ethanol series (70%, 80%, 90%, 100% at 2 minutes each). Allow to air dry at room temperature.
   70% Formamide, 2X SSC:
   175 ml 100% formamide
   25 ml 20X SSC
   50 ml water
   pH 7.0 with HCl; store at 4°C in dark.
3. Quickspin probe and transfer 3 µl to a new microfuge tube.
4. Denature probe at 70°C for 10 minutes.
5. Preanneal probe mix at 37°C for 20 minutes and quickspin.
6. Apply 2 to 4 µl of preannealed probe mix to chromosome slide. Cover with a coverslip and seal the edges with rubber cement.
7. Place the slides in a moist chamber and incubate at 37°C overnight.

**D. Washing and Signal Detection**

1. Remove rubber cement with a needle and rinse slides in 2X SSC until coverslips come off.
2. Wash slides three times in 50% formamide, 2X SSC at 40-45°C for 5 minutes.
3. Wash slides three times in 4X SSC, 0.05% Tween20 at room temperature for 2 minutes.
4. Wash slides once in 4X SSC at room temperature for 2 minutes.
5. Add 0.2 µl Avidin-FITC in 200 µl PNM Buffer to each slide. Coverslip and incubate in a moist chamber for 30 minutes.
6. Wash slides three times in 4X SSC, 0.05% Tween20 at room temperature for 2 minutes.
7. Wash slides once in 4X SSC at room temperature for 2 minutes.
8. Add 2 µl Biotinylated Anti-Avidin in 200 µl PNM buffer to each slide. Coverslip and incubate for 30 minutes.
9. Wash slides three times in 4X SSC, 0.05% Tween20 at room temperature for 2 minutes.
10. Wash slides once in 4X SSC at room temperature for 2 minutes.
11. Add 0.2 µl Avidin-FITC in 200 µl PNM buffer to each slide. Coverslip and incubate for 30 minutes.
12. Wash slides three times in 4X SSC, 0.05% Tween20 at room temperature for 2 minutes.
13. Wash slides once in 4X SSC at room temperature for 2 minutes.
14. Apply 10-20 µl of DAPI/anti-fade solution to each slide and coverslip. Keep slides in a dark box at 4°C.

**E. Solutions**

1. PN buffer

   0.1 M $Na_2HPO_4$ X 2 $H_2O$

   Adjust pH to 8.0 with 0.1 M monobasic $NaH_2PO_4$ X $H_2O$

2. PNM buffer

   5% dry milk in 0.1 M PN buffer pH 8.0. Dissolve overnight at 37°C then centrifuge in microfuge tube at max speed for 10 minutes. Divide supernatant into 500-1000 µl aliquots and freeze.

3. 20X SSC

   88 g Sodium Citrate

   175 g Sodium Chloride

   Mix well and filter with 0.2 µm bottle top filter.

# APPENDIX B

## RADIATION HYBRID (RH) MAPPING PROTOCOL

**Chowdhary et al. (2002, 2003)**

1. Optimize primers in horse genomic DNA ensuring that hamster genomic DNA is not amplified and primer amplification is horse specific.
2. Use the 5000rad whole-genome RH panel comprised of 92 hybrid cell lines to type each primer pair by PCR. Make sure to include a 'no-DNA' control as well as horse and hamster genomic DNA for positive controls.
3. Resolve PCR products on a 2.5% agarose gel (containing 0.25 µg/ml ethidium bromide).
4. Manually score the gel results using 0, 1 & 2 to signify bands are either present (0), absent (1) or indistinguishable (2) for each lane of the gel and record results in Microsoft Excel spreadsheet.
5. Repeat steps 2 through 5 to repeat the RH panel typing and confirm results.
6. Use two-point linkage analysis to obtain nearest marker information with 1000:1 odds (or LOD 3).

# APPENDIX C

## *IN SITU* HYBRIDIZATION (ISH) PROTOCOL

### A. Probe Transcription

1. Place (anti-sense) DNA template gel chunks (bands cut from low-melt gel) at 70°C for 10 minutes, then transfer to 37°C for use.
2. Mix the following reaction in order at room temperature for each sample:

| Reagent | Volume |
|---|---|
| DEPC water | 1.7 µl |
| 5X Transcription Buffer | 4.0 µl |
| 1 M DTT | 0.2 µl |
| 33 uM ACG nucleotides | 0.3 µl |
| 100 uM UTP | 0.5 µl |
| RNasin | 0.8 µl |
| 4 µl $^{35}$S UTP | 4.0 µl |
| DNA from gel chunk | 4.0 µl |
| RNA Polymerase enzyme | 1.0 µl |
| Total | 20.0 µl |

3. After addition of gel chunk, the reaction should stay at 37°C. Mix the reaction thoroughly with pipette and incubate for 1 hour.
4. Remove 1 µl of the transcription reaction and add 9 µl of formamide. Heat at 70°C for 10 minutes then load samples on the probe test gel. Load 3 µl of dye marker in an extra well.
5. Run gel at 40 mAmps for 45 minutes.
6. Allow transcription reaction to continue for another hour.
7. Expose gel to XAR film for 1 hour or overnight.
8. Add 1 µl of RQ1 DNase to each transcription reaction. Vortex samples and incubate at 37°C for 15 minutes.
9. Freeze the probes at –80°C overnight.

### B. Probe Cleanup

1. Develop film and proceed with probe cleanup if all probes appear to have incorporated radioactivity.
2. Add 30 µl sterile water to each probe.
3. Phenol chloroform isoamyl (PCI) extract with 50 µl of PCI (pH 5.0). Vortex and centrifuge at 10,000 rpm for 4 minutes.
4. Remove upper aqueous layer and add 50 µl of chloroform isoamyl (CI). Vortex to mix and centrifuge at 10,000 rpm for 4 minutes.
5. Remove upper aqueous layer by pipetting.
6. Use Roche RNA MiniQuick Spin Columns (Roche, Switzerland) to cleanup probes. (Follow instructions to resuspend column and spin samples.)
7. Check probe counts using 1 µl of probe in scintillation fluid.
8. Heat probes at 68°C before use.

**C. Slide Preparation**

1. Deparaffinize the sections by taking the slides through the following steps:

| | |
|---|---|
| CitriSolv™ (Fisher Scientific) | 2 minutes |
| 100% Ethanol | 2 minutes |
| 100% Ethanol | 2 minutes |
| 95% Ethanol | 2 minutes |
| 70% Ethanol | 2 minutes |
| 50% Ethanol | 2 minutes |

2. Fix the sections in 4% paraformaldehyde for 10 minutes at 4°C.
3. Wash slides in 0.5X SSC for 5 minutes at room temperature.
4. Soak the slides in Proteinase K (20 µg/ml in RNase buffer) for 10 minutes at room temperature (RT).
5. Wash slides in 0.5X SSC for 10 minutes at room temperature.
6. Preheat Hybridization Buffer to 55°C.
7. Blot the slides dry and place them in tupperware or pyrex containers resting on plastic pipettes. A paper towel soaked in box buffer is placed on the bottom of the container.
8. Circle tissue sections with rubber cement to form a reservoir for the probes.
9. 120 µl is added to each section containing 500,000 counts of probe, 10% volume of 1mM DTT, 2 µl/section of yeast tRNA and hybridization buffer. The probe and yeast tRNA mixture is heated at 70°C for 5 minutes then placed on ice.
10. The container with the slides is covered and the slides incubate overnight at 55°C.

**D. Slide Washing**

1. Heat a shaking water bath to 55°C. Make 2 liters of 0.1X SSC, 10 mM 2-mercaptoethanol (2-ME), 1 mM EDTA buffer and place slides in 55°C water bath.
2. Slides are placed in a rack and washed as follows (**the waste is radioactive--use proper containers and disposal procedures):**
   A. 2X SSC with 10 mM 2-ME + 1 mM EDTA, 10 min., RT
   B. Repeat A
   C. 20 µg/ml RNase A in RNase Buffer, 30 min., RT
   D. 2X SSC with 10 mM 2-ME + 1 mM EDTA, 10 min., RT
   E. Repeat D
   F. 0.1X SSC with 10 mM 2-ME + 1 mM EDTA, 1 hour, 55°C, gently shaking
   G. Repeat F
   H. 0.5X SSC, 10 min., RT
   I. Repeat H
3. Dehydrate slides by placing through ethanol washes as follows:
   50% Ethanol + 0.3 M $NH_4Ac$, 2 min., RT
   70% Ethanol + 0.3 M $NH_4Ac$, 2 min., RT
   90% Ethanol + 0.3 M $NH_4Ac$, 2 min., RT
4. Remove the rubber cement by lifting the edge with a needle.
5. Blot slides dry on the edge and dry in racks on a tray for 1-to-3 hours at 37°C.
6. Line slides up in a film cassette. Tape down the edges. Expose to film 3-to-4 days at room temperature with the cassette wrapped in foil.

**E. Slide Dipping**

1. If the film shows that the slides look "good," then the slides are dipped in Kodak NTB2 nuclear emulsion in the dark room.
2. Emulsion is diluted 1:1 with water. Emulsion should be at 42ºC in a water bath and poured into dipmizer.
3. First a blank slide is dipped and developed to test the emulsion quality (Developer 5 min., water 30 sec., Fixer 5 min. and running water 5 min.).
4. Dip experimental slides in emulsion at 42ºC and lean them at a diagonal on a slide box to dry. Transfer the slides to a slide box containing fyrite wrapped in a kimwipe.
5. Dry slides in the bread box for 2 hours without the lid.
6. Wrap slide boxes with foil and place at 4ºC until time to develop.

**F. Slide Development**

1. Place slides in Kodak Developer diluted 1:1 with water and at 15ºC for 4 minutes in the dark room.
2. Place slides in distilled water for 30 seconds.
3. Place slides in Kodak Fixer with Hardener for 5 minutes.
4. Place slides under running water for 5 minutes.
5. Slides can be brought out of the dark room but must be kept wet.
6. Stain slides using 1% Toludine Blue stain (made in Boric Acid) for 4 minutes.
7. Wash slides with water for 30 seconds.
8. Place slides in 50% Ethanol for 3 minutes.
9. Place slides in 70% Ethanol for 2 minutes.
10. Place slides in 95% Ethanol for 2 minutes.
11. Place slides in 100% Ethanol for 1 minutes.
12. Place slides in Xylene for 1 minute.
13. Use Permount to cover slip slides.
14. Allow slides to dry for 3 days then clean up slides using Xylene.

**G. Recipes for *in situ* hybridization solutions**

1. 20X SSC

   173 g Sodium Chloride
   88.2 g Sodium Citrate
   Bring up to 1 liter with distilled water. Add 1 ml Diethyl Pyrocarbonate (DEPC) and shake vigorously. Place at 37ºC overnight. Autoclave for 40 minutes.

2. 0.5X SSC

   25 ml 20X SSC
   975 ml DEPC water

3. 4% paraformaldehyde (200 ml)

   In a fume hood, heat 100 ml of nanopure water in a beaker and stir in 8 g of paraformaldehyde. Add 1-2 drops of 2 M NaOH and the solution should clear. Remove from heat and add 66 ml of 3X PBS. Adjust pH to 7.4 with HCl and bring up to 200 ml with distilled water.

4. 2X SSC with 10 mM 2-ME + 1 mM EDTA
   100 ml 20X SSC
   77 µl 13 M 2-ME
   2 ml 0.5 M EDTA
   Bring to 1 liter with DEPC water.

5. 0.1X SSC with 10 mM 2-ME + 1 mM EDTA
   10 ml 20X SSC
   154 µl 13 M 2-ME
   4 ml 0.5 M EDTA
   Bring to 2 liters with DEPC water.

6. RNase Buffer (500 mM NaCl, 10 mM Tris) pH 8.0
   50 ml 5 M NaCl
   5 ml 1 M Tris, pH 8.0
   445 ml Nanopure water

7. Box Buffer (4X SSC + 50% formamide)
   100 ml 20X SSC
   250 ml formamide
   150 ml DEPC water

8. Hybridization Buffer
   25 ml Formamide
   6 ml 2.5 M NaCl
   1 ml 1 M Tris, pH 8.0
   0.5 ml 0.5 M EDTA
   1 ml 0.5 M sodium phosphate, dibasic
   5 g Dextran sulfate
   1 ml 50X Denhardt's
   5 ml DEPC water
   Incubate at 37ºC on a rocker for 4 hours to dissolve. Add 1/10 volume 1M DTT immediately prior to use.

9. 50X Denhardt's (to 500 ml with DEPC water)
   5 g Ficoll 400
   5 g Polyvinylpyrrolidone
   5 g BSA (Pentax Fraction V)

# APPENDIX D

## RNA EXTRACTION PROCEDURE

**** Always wear gloves!**

1. Pipette 3 ml of TriPure (Roche, Switzerland) into 50 ml conical tubes. One tube for each tissue sample. Keep tissue and samples on ice.
2. Place vials of tissue on ice. Transfer ~ 0.3 g of tissue to designated 50-ml conical tube.
3. Record the weight of each amount of tissue used.
4. Wash tissue homogenizer with ddH2O thoroughly before use.
5. Homogenize each tissue in 50-ml conical tubes for 30 seconds. Go back through samples a second time and homogenize again for 30 seconds.
   ***** Clean homogenizer between each sample with two different containers of ddH2O. Wipe off excess water with Kimwipes. ****** Keep tissue on ice.
6. Can keep tissue at 4°C overnight at this point and finish extraction procedure the next day.
7. Let samples sit at room temperature for 5 minutes.
8. Under the fume/solvent hood add 600 µl of chloroform to homogenized tissue. Vortex and transfer each sample to RNAse Zap treated plastic tubes properly labeled. (Color change seen from clear to opaque.)
9. Incubate samples for 2-to-15 minutes at room temperature.
10. Spin for 15 minutes at 12,000 rpm at 4°C.
11. Remove top layer of RNA with pipette and transfer to new plastic tube (treated with RNAzap). Stay away from the white DNA layer.
12. Add 1.5 ml of isopropanol to each sample. Vortex and incubate for 10 minutes at room temperature.
13. Centrifuge at 12,000 rpm for 10 minutes at 4°C.
14. Remove supernatant and discard.
15. Add 3 ml of 70% ethanol made with DEPC ddH2O.
16. Vortex until pellet floats. Spin at 12,000 rpm for 10 minutes at 4°C.
17. Remove Ethanol and discard. Allow pellets to dry. Should use Kimwipe tissue paper to wipe tube dry.
18. Resuspend pellets in 100 µl of DEPC ddH2O.
19. Incubate at 68°C for 10 minutes.
20. Collect volume into 1.5 ml microfuge tubes. Aliquot 3 µl of sample to run 1.5% Agarose denaturing gel and 5 µl of sample diluted in 995 µl of DEPC ddH2O to spec concentration.
21. Store RNA samples at –80°C. Minimize number of thaws and time spent on ice to prevent degradation.

# APPENDIX E

## RIBONUCLEASE PROTECTION ASSAY (RPA) PROTOCOL

### A. Gel Preparation

1. Clean one short and one long sequencing plate by washing with Alconox detergent and rinsing with tap water.
2. Clean plates with RNazap (Ambion, Inc., Austin, Texas).
3. Clean plates with DEPC water.
4. Clean plates with 100% ethanol.
5. Clean plates with DEPC water.
6. Allow plates to dry.
7. Wipe short plate with Sigmacote (Sigma) and allow to dry.
8. Assemble plates with spacers on each side and rubber clamp around outside edge.
9. Prepare gel using the following recipe:

    8.85 ml 40% Acrylamide
    9.31 ml 2% Bis-acrylamide
    7.45 ml 10X TBE
    35.82 g Urea

    Mix the above ingredients with stir bar in RNazap treated glassware. Apply low heat and spin until in solution. Bring volume to 75 ml with DEPC water. Place gel mixture on ice and allow to cool. When ready to pour gel, add 450 µl of 10% Ammonium Persulfate (freshly made) and 60 µl of Temed. Gently swirl mixture and pour gel.
10. Make sure no bubbles are present in the gel. Place the comb in between the plates and clamp the comb and the plates together.
11. Allow the gel to solidify for two hours before removing the clamps and the comb.
12. Pre-run the gel in 1X TBE buffer at 50 watts for 20 minutes before loading the sample probes for gel purification.

### B. Probe Synthesis

1. Thaw the frozen reagents from Ambion MAXIscript *in vitro* transcription kit (cat# 1312; Ambion, Inc., Austin, Texas).
2. Assemble the following transcription reaction at room temperature. Heat DNA template gel chunks at 70°C for 10 minutes, then transfer to 37°C for sample removal.

| Component | Sample (µl) | Century Marker (µl) |
|---|---|---|
| cDNA Template | 1.0 | 1.0 |
| Nuclease-free water | 15.25 | 10.0 |
| 10X Transcription Buffer | 3.5 | 2.0 |
| 10 mM ATP | 1.75 | 1.0 |
| 10 mM CTP | 1.75 | 1.0 |
| 10 mM GTP | 1.75 | 1.0 |
| 10 mM UTP | 0 | 0 |
| 10 mM 32P UTP | 8.0 | 2.0 |
| T7 RNA Polymerase | 2.0 | 2.0 |
| TOTAL | 35 µl | 20 µl |

3. Mix all reactions gently. Incubate reactions at 37°C for one hour.
4. Add 1.0 µl of DNase 1, mix well and incubate at 37°C for 15 minutes.
5. Add 1.0 µl of 0.5M EDTA and 30 µl of gel loading dye. For marker, remove 1.5 µl of reaction and add 8 µl of gel loading dye.
6. Heat samples at 90°C for 3 minutes before loading on pre-run gel.
7. Run gel at 50 watts for approximately one hour.
8. Gently pull plates apart and cover gel on long plate with saran™ wrap. Place XAR film on top of saran™ wrap and expose for 2-5 minutes.
9. Use film to mark probe bands on back of glass plate.
10. Cut out probe bands from gel and place in 50 ml tube containing 300 µl of Elution buffer.
11. Place probes at 37°C for at least one hour.
12. Count 1 µl of probe using Beta Counter.

**C. Ribonuclease Protection Assay**

1. Thaw the frozen reagents from the Ambion RPA III kit (cat# 1414; Ambion, Inc., Austin, Texas).
2. Mix 20 µg of sample RNA and 100,000 counts of labeled probe.
3. Set up yeast A and yeast B control tubes containing 4 µl of yeast RNA and the same volume of probe as the RNA samples.
4. Co-precipitate the probes and sample RNA using 1/10 reaction volume of $NH_4OAc$ and 2.5 volumes of cold 100% ethanol.
5. Place tubes in –20°C for at least 15 minutes.
6. Centrifuge samples at max speed (10,000 rpm) for 15 minutes.
7. Pipette off supernatant and air dry the pellets for at least 5 minutes.
8. Resuspend pellets with 10 µl Hybridization buffer and quick-spin tubes.
9. Heat samples at 90-95°C for 3-4 minutes.
10. Incubate samples at 42°C overnight.
11. Make 1:100 dilution of RNase A/T1 in RNase Digestion III buffer calculating enough mix to add 150 µl to each sample EXCEPT for the yeast B control.
12. Quick-spin the samples and add 150 µl of the above mixture.
13. Vortex and quick-spin samples.
14. Incubate samples at 37°C for 30 minutes.

15. Add 1µl of yeast RNA to all samples.
16. Add 225 µl RNase Inactivation/Precipitation III Solution to each sample.
17. Add 75 µl of cold 100% ethanol to each sample.
18. Place samples at –20°C for 15 minutes.
19. Centrifuge samples at maximum speed (10,000 rpm) for 15 minutes at 4°C.
20. Remove supernatant and allow samples to dry very well.
21. Resuspend samples with 6 µl of gel loading dye.
22. Heat all samples (including the marker) at 90°C for 3 minutes.
23. Load samples on 5% acrylamide gel and run gel at 50 watts for 2 hours.
24. Separate plates and place 3 mm Whatman filter paper (cat# 28458-005; VWR, West Chester, PA) cut to the gel size on top of the gel. Roll a 50 ml tube on top of the Whatman paper until the gel can be pulled off of the plate.
25. Cover the gel and Whatman paper with saran™ wrap and dry the gel on a pre-warmed apparatus for 15 minutes.
26. Tape the gel into a film cassette and expose to XAR film for 1-3 days at –80°C until a good image is acquired.

# APPENDIX F

## SLOT BLOT PROCEDURE FOR mRNA ANALYSIS

### A. Membrane Preparation

1. Clean all components of slot blot apparatus with RNAzap (Ambion). Rinse well with DEPC water.
2. Use 20 µg of RNA per slot. (If RNA sample is too dilute, then precipitate RNA using 1/10 volume 3M NaOAc and 3 volumes 100% EtOH. Mix well and place at –80ºC for 20 minutes. Centrifuge at 10,000 x g for 10 minutes at 4ºC. Pour off supernatant. Quick spin and pipet off remaining EtOH. Reconstitute pellet in 10 µl of DEPC water.)
3. Wet a piece of nylon transfer membrane (Schleicher and Schuell, Keene, NH) briefly in water and soak in 20X SSC for 1 hour at room temperature.
4. Place two sheets of Whatman 3mm paper pre-wet with 20X SSC on top of the base (vacuum unit) of the slot blot apparatus. Place the wet nitrocellulose on top of the blotting paper, and smooth out any air bubbles. Clamp on the top of the apparatus and hand tighten screws in a diagonal fashion if using Bio-RAD.
5. Fill all slots with 10X SSC and apply gentle suction until the fluid has passed through all slots. Turn off the vacuum and refill each slot. NOTE: Filter 20X SSC and 10X SSC solutions with a 0.2 µm filter to prevent clogging.

*Preparation of RNA Samples*

6. Add the following to each 10 µl (20 µg) of RNA sample. Use DEPC water if necessary to q.s. each sample to 10 µl total volume.

| | |
|---|---|
| Formamide (100%) | 20 µl |
| Formaldehyde (37%) | 7 µl |
| 20X SSC | 2 µl |

7. Mix well and denature samples at 70ºC for 15 minutes. Cool on ice for 5 minutes.
8. Add 2 volumes (80 µl) of 20X SSC to each sample. Mix well and centrifuge briefly. Keep on ice.
9. Remove 10X SSC from slot blot apparatus with gentle suction. Turn off vacuum.
10. Load samples into slots and apply gentle suction. After samples have passed through, rinse each slot twice with 10X SSC. Continue suction until all wells are dry.
11. Remove filter from apparatus and allow to dry completely at room temperature. Bake at 80ºC for 2 hours. Store at –20ºC for up to 6 months.

**B. Northern and Slot Blot Hybridization with cRNA Probes**

1. Make hybridization buffer:

| Concentration | for 100 ml |
|---|---|
| 5X SSC | 25 ml of 20X SSC |
| 5X Denhardt's | 10 ml of 50X Denhardt's |
| 50% Formamide | 50 ml Formamide |
| 1% SDS | 10 ml of 10% SDS |
| DEPC water | 5 ml DEPC water |
| 100 µg/ml Salmon Sperm DNA | 2 ml of 10 mg/ml stock |

NOTE: Before use, denature salmon sperm DNA for 5-10 minutes at 95-100ºC. Add directly to hybridization buffer before use.

2. Wet nylon membrane briefly with 2X SSC.
3. Place membrane in hybridization buffer (~20 ml for medium tubes) in roller tubes. Prehybridize at 55ºC for 1 hour.
4. Synthesize antisense cRNA probe from antisense DNA with Ambion Maxiscript kit.

| Component | Amount/probe |
|---|---|
| Nuclease-free water | qs to 20 µl |
| DNA template | 1 µg |
| 10X Transcription Buffer | 2 µl |
| 10 mM ATP | 1 µl |
| 10 mM CTP | 1 µl |
| 10 mM GTP | 1 µl |
| 10 mM UTP | ---- |
| 32P labeled UTP | 5 µl |
| RNA Polymerase | 2 µl |

Mix all ingredients above in 1.5-ml microfuge tubes for each probe needed. Incubate probes to synthesize at 37ºC for 1 hour. Add 80 µl of DEPC water to each probe. Extract with equal volume of Phenol, Chloroform, Isoamyl Alcohol (24:25:1). Vortex and spin for 2 minutes at max speed. Transfer top layer to new microfuge tube. Add equal volume of chloroform, vortex and spin for 2 minutes at max speed. Transfer top layer to new microfuge tube. Column purify each probe and count 2 µl on liquid scintillation counter.

5. Add 500 µl of hybridization buffer to microfuge tube containing desired amount of radiolabeled antisense cRNA probe. Usually desire 1 x $10^6$ cpm/ml hybridization buffer.
6. Denature probe at 70ºC for 5-10 minutes. Place on ice for 2 minutes.

7. Add probe to hybridization buffer in roller tube and hybridize overnight (12-16 hours) at 55ºC. Use as little of the hybridization buffer as necessary to ensure complete coverage of the blot during hybridization. For medium tubes, 10-to-12 ml should be enough—remove extra buffer before addition of probe.
8. Prepare washes in advance and warm to desired temperature.
9. Wash the blot with 50 ml of 2X SSC/0.1% SDS at room temperature for 20 minutes.
10. Wash the blot with 50 ml of 1X SSC/0.1% SDS at 55ºC for 20 minutes.
11. Wash the blot 3 times with 50 ml of 0.1X SSC/0.1% SDS at 68ºC for 20 minutes each.
12. Wash blot 3 times with 2X SSC for 5 minutes each (to remove SDS and fomamide).
13. Incubate blot in 2X SSC containing 1 µg/ml RNAse A for 15 minutes at room temperature.
14. Wash blot twice in 0.2X SSC/0.1% SDS at 55ºC for 15 minutes each.
15. Wrap blot in saran wrap.
16. Expose blot to autoradiography. Either use phosphorimager or expose to film overnight at –80ºC.

**C. Solutions**

1. 50X Denhardt's (500 ml)
   5 g Ficoll 400
   5 g Polyvinylpyrrolidone
   5 g BSA (Pentax Fraction V)
   DEPC Water

2. 20X SSC (1L)
   88 g Sodium Citrate
   175 g Sodium Chloride
   Mix well and filter with 0.2 µm bottle top filter.

3. 10X SSC (1L)
   44 g Sodium Citrate
   87.5 g Sodium Chloride
   Mix well and filter with 0.2 µm bottle top filter.

4. 2X SSC (500 ml)
   50 ml of 20X SSC bring to volume with DEPC water.

5. 10% SDS (250 ml)
   25 g Sodium Dodecyl Sulfate (Laural Sulfate)
   Mix and bring to volume with DEPC water.

6. 2X SSC/0.1% SDS (500 ml)
   50 ml of 20X SSC
   500 µl 10% SDS
   Bring to volume with DEPC water.

7. 1X SSC/0.1% SDS (500 ml)
   25 ml of 20X SSC
   500 µl 10% SDS
   Bring to volume with DEPC water.

8. 0.1X SSC/0.1% SDS (500 ml)
   2.5 ml of 20X SSC
   500 µl 10% SDS
   Bring to volume with DEPC water.

9. 0.2X SSC/0.1% SDS (500 ml)
   5 ml of 20X SSC
   500 µl 10% SDS
   Bring to volume with DEPC water.

10. RNAse A
    10 mM Tris
    15 mM NaCl
    Mix and boil for 10 minutes. Store at –20ºC.

# APPENDIX G

## INSULIN-LIKE GROWTH FACTOR TYPE I (IGF-I) ASSAY PROTOCOL

## FOR BOVINE SERUM

### Reagent Preparation

1. IGF-I Assay Buffer
   0.40 g Protamine (grade II) $SO_4$ (Sigma P-4380)
   8.28 g Sodium phosphate (monobasic) (Sigma S-9638)
   1.0 ml Tween 20 (Sigma P-1379)
   0.40 g Sodium azide (Sigma S-2002)
   7.44 g EDTA

   Mix above reagents in double-distilled water ($ddH_20$). pH solution to 7.5 with NaOH and bring volume to 2.0 liters. Store solution at 4ºC for one month.
   **(Caution: Sodium azide is highly toxic.)**

2. 1M Glycine
   75.07 g Glycine (Sigma S-7126)
   Mix glycine in approximately 850 ml $ddH_20$. Using 38% HCl, adjust pH of solution to 3.2 and bring volume to 1.0 liter. Make fresh 1M glycine for each extraction and store at 4ºC.

3. 0.5N NaOH
   Add 50 ml of 2.5N NaOH to 200 ml $ddH_20$ (1:5 dilution) or dissolve 5.0 g NaOH pellets into 250 ml $ddH_20$. Store at 4ºC.

4. 12.5% Polyethylene Glycol (PEG)
   250 g Carbowax PEG 8000 (Fisher P1567-500)
   Mix PEG in approximately 1800 ml $ddH_20$, cover and mix at room temperature until solution is clear (2 hours). Adjust pH of solution to 8.6 and bring volume to 2.0 liters. Store at 4ºC for up to 3 months.

5. Primary Antibody (Bovine IGF-I antibody for RIA)
   (Source: A. F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, 1000 West Carson St., Torrance, CA 90509; Phone #: 310.222.3537)

   Antibody comes lyophilized at a 1:10 dilution in PBS. Use antibody at a final dilution of 1:120,000 in IGF-I Assay Buffer (7.5 µl stock into 90 ml IGF-I Assay Buffer). Prepare fresh daily at least one hour before use and store at 4ºC.

6. Secondary Antibody (GARGG)
   (Source: Calbiochem, San Diego, CA; Goat Anti-Rabbit SA IgG)

Add 667 µl of stock GARGG to 39.33 ml IGF-I Assay Buffer (1:60 dilution). Prepare fresh daily and store at 4ºC.

7. Normal Rabbit Serum (NRS)
   Prepare at 1:100 dilution in IGF-I Assay Buffer (500 µl stock into 49.5 ml Buffer).

8. [$^{125}$I] Tracer
   (Source: ICN Biomedicals Inc., Cat# 68128)

   Calculation of required activity:
   1µCi isotope = 22.20 x $10^6$ dpm.
   2.22 x $10^6$ dpm = 1.665 x $10^6$ cpm (At 75% counting efficiency estimate [$^{125}$I]).

$$\frac{\text{(n) RIA tubes}}{1} \times \frac{\text{21,000 cpm}}{\text{tube}} \times \frac{\text{µCi}}{\text{1,665,000 cpm}} = \text{Required Activity (µCi)}$$

   = approx. 15 µCi/1000 RIA tubes

   Prepare trace at RIA working dilution of 21,000 cpm/100 µl. This would result in, for example, 60 µCi $^{125}$I IGF-1 trace being diluted out to 500 ml RIA working stock:

$$\frac{\text{60 µCi}}{1} \times \frac{\text{1,665,000 cpm}}{\text{µCi}} \times \frac{\text{RIA tube}}{\text{21,000 cpm}} \times \frac{\text{1 ml}}{\text{10 RIA tubes}} = \text{500 ml trace}$$

   Prepare and store in an appropriately labeled HD polypropylene bottle wrapped with lead-foil shielding (preferably set in a vessel of greater volume for stability, handling safety and spill control).

   Survey and thaw raw trace shipment under hood. Measure out and record approximately 75 % of the tracer's expected final volume of RIA buffer. Pour back and hold ~10 ml of measured buffer volume. Using this volume, and a 1 cc syringe w/ 20 GA needle, suspend and aspirate trace into 1.0 ml. Carefully transfer this, liquid to liquid, into the storage bottle. Rinse tracer's pig, through septum, with at least 5 x 1.0 ml aliquots of same buffer, transferring each to storage bottle using the same syringe. Add unused buffer remainder to storage bottle. Let dilution set for at least two hours before counting (n = 4) x 100 µl aliquots x 1 min. Calculate the final working dilution as above. Make final dilution and store, preferably overnight, before use.

9. IGF-I Standards
   Absolute range of IGF-1 standards @1:200 sample dilution is 19.54 through 5000 ng/ml serum. (Dilutions are 1:100 at sera extraction, and 1:2 in RIA; final = 1:200.)

Expected biological range should be approximately 40 to 250 ng/ml sera, therefore most samples should be represented by the range between the 0.98 and 1.56 ng/ml standards.
hIGF-1, BIO: Lot #01, sample #1168, 134 µg/vial, lyophilized.
Source: A.F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, 1000 West Carson St, Torrance CA 90509 phone 310.222.3537, email parlow@humc.edu

Reconstitute lyophilized standard stock with 1.00 ml dd$H_2O$ (IGF-1 STD Stock I). Note: this resulted in a previous shipment of this specific standard (sample #1168) having a concentration of 134 µg/ml (vial's specific mass listed on same by FJP).

Construct 1 µg/ml IGF-1 STD Stock II. Transfer 74.63 µl (i.e. 10 µg) to a 10 ml volumetric containing approximately 8.0 ml IGF-1 RIA buffer (liquid to liquid, using a Hamilton syringe). Bring to volume and allow for equilibration. Aliquot and freeze if not used immediately.

Prepare serial dilutions of IGF-1 standards fresh for each RIA series. Add 625 µl of 1 µg /ml IGF-1 stock II to a 25 ml volumetric containing approximately 20 ml IGF-1 RIA buffer. Use liquid-to-liquid transfer, and allow for equilibration. The resulting STD A = 25 ng/ml.

Continue preparation of serial dilutions by mass, rather than by volume. Label (n = 8) 50 ml PP conical-bottom centrifuge tubes for the following array of standards. Tare each tube on a three-place balance and load each with exactly 12.00 g of IGF-1 RIA buffer. Transfer exactly 12.00 g of STD A to the STD B tube (12.5 ng/ml). Mix by gentle vortexing and/or inversion, then allow to equilibrate for a minute or two before continuing with the next 1:1 dilution (STD C = 6.25 ng/ml). Continue serial dilutions through STD I (0.098 ng/ml). Aliquot each IGF-1 standard at 1.0 or 2.0 ml and freeze sets until use. Prepare fresh for each RIA series.

IGF-I Standards chart:

| | ng/ml | ng/tube | equivalence |
|---|---|---|---|
| IGF-1 STD | at RIA | at RIA | ng/ml sera |
| STD A | 25.000 | 2.500 | 5000.00 |
| STD B | 12.500 | 1.250 | 2500.00 |
| STD C | 6.250 | 0.625 | 1250.00 |
| STD D | 3.125 | 0.313 | 625.00 |
| STD E | 1.563 | 0.156 | 312.50 |
| STD F | 0.781 | 0.078 | 156.25 |
| STD G | 0.391 | 0.039 | 78.13 |
| STD H | 0.195 | 0.020 | 39.06 |
| STD I | 0.098 | 0.010 | 19.53 |
| STD J | 0.000 | 0.000 | 0.00 |

10. IGF-1 Composite Pools for RIA:

For verification of inter- and intra-RIA performance over the expected biological IGF-1 concentration range, construct a "normal" pool and a "high" pool from a composite sub-set of acidified serum samples.

Pool (n = 30) 200 µl aliquots from a random set of acidified serum samples. Pipette 3.00 ml of this to a 13 x 100 mm PP culture tube (to be used for the "high" pool preparation). Aliquot remainder of "normal" pool at 500 µl, freeze and store until use.

For the "high" pool, prepare a 100 ng/ml IGF-1 stock through a 1:10 dilution of 1 µg /ml IGF-1 Standard Stock II. Pipette 20 µl of the 100 ng/ml stock into 2980 µl of the "normal" pool aliquot to produce the "high" pool stock (e.g. back-pipette 20 µl from the 3.0 ml and replace). This results in the addition of 0.667 ng/ml at RIA or, after accounting for the 1:200 final sample dilution following RIA, a delta at RIA of 133.3 ng/ml. (e.g. "normal" IGF-I concentration plus 133 ng/ml at the serum level). Vortex and aliquot "high" pool at 500 µl, freeze and store until use.

**IGF-I Assay Protocol:**

**A. Acidification of Samples**

1. Pipette 10 µl of each bovine serum sample into 12X75 polypropylene culture tubes. (Polypropylene tubes must be used due to low pH. Number tubes in odd numbers so that samples can be assayed in duplicate.)
2. Add 400 µl of 1M glycine to each sample.
3. Add 500 µl of IGF-I Assay Buffer to each sample.
4. Cap tubes and incubate in 37ºC water bath for 48 hours.
5. Add 90 µl of 0.5N NaOH to all samples and vortex to mix. (Continue assay immediately. Samples are diluted 1:100 and final calculations will need to be multiplied by 100.)

**B. Assay Procedure**

1. Each assay should include at least triplicate tubes of total (T), non-specific binding (NSB), zero tubes ($B_0$), standards, and pools. Single acidified samples should be pipetted into duplicate for the RIA.
2. Pipette 400 µl of IGF-I Assay Buffer into NSB tubes.
3. Pipette 300 µl of IGF-I Assay Buffer into $B_0$ tubes.
4. Pipette 200 µl of IGF-I Assay Buffer into standard tubes.
5. Pipette 250 µl of IGF-I Assay Buffer into all sample tubes and pools.
6. Add 100 µl of each standard to each designated standard tube.
7. Add 50 µl of each acidified serum sample into each designated tube pair.
8. Add 50 µl of acidified pools into control pool tubes.
9. Pipette 100 µl of primary antibody to all tubes except NSB and T.
10. Carefully shake tubes to mix and cover with parafilm.
11. Incubate for 24 hours at 4ºC.
12. Pipette 100 µl of [125I]-IGF-I Tracer to all tubes.

13. Cover tubes with parafilm and shake the tubes carefully to mix.
14. Incubate for 16 hours at 4ºC.
15. Pipette 50 µl of NRS to all tubes except totals.
16. Pipette 50 µl of GARGG to all tubes except totals.
17. Pipette 300 µl of PEG to all tubes except totals.
18. Carefully shake tubes to mix and cover with parafilm.
19. Incubate tubes at room temperature for 30 minutes.
20. Centrifuge tubes at 3000 rcf for 25 minutes at 4ºC.
21. Decant tubes (except totals) immediately into radioactive waste container.
22. Allow tubes to remain upside down on absorbent towels for 5 minutes.
23. Remove all visible droplets by tapping tube bottoms.
24. Count tubes on Beckman gamma counter for 1 minute per sample.
25. Use AssayZap to calculate concentrations of unknowns in comparison to a known standard curve.

# APPENDIX H

## TESTOSTERONE RADIOIMMUNOASSAY (RIA) PROTOCOL

1. Thaw frozen serum or EDTA plasma samples and place on ice.
2. Pipet blood samples into 12X75 polypropylene tubes in duplicate.
3. Bring volume in each tube to 500 µl with PBSG.
4. Pipet 500 µl of standards in duplicate starting at 8000 pg of testosterone per tube and continuing to 3.9 pg per tube according to the following table. The total, non-specific binding (NSB) and 0 tubes only receive PBSG buffer.

| **Standard** | **PBSG Buffer** | **Sample** |
|---|---|---|
| Total | 800 µl | 0 µl |
| Non-specific Binding (NSB) | 600 µl | 0 µl |
| 0 pg/tube | 500 µl | 0 µl |
| 3.9 pg/tube | 0 µl | 500 µl |
| 7.9 pg/tube | 0 µl | 500 µl |
| 15.6 pg/tube | 0 µl | 500 µl |
| 31.25 pg/tube | 0 µl | 500 µl |
| 62.5 pg/tube | 0 µl | 500 µl |
| 125 pg/tube | 0 µl | 500 µl |
| 250 pg/tube | 0 µl | 500 µl |
| 500 pg/tube | 0 µl | 500 µl |
| 1000 pg/tube | 0 µl | 500 µl |
| 2000 pg/tube | 0 µl | 500 µl |
| 4000 pg/tube | 0 µl | 500 µl |
| 8000pg/tube | 0 µl | 500 µl |

5. Add 100 µl of testosterone antibody (Colorado State University) to each tube except for the total and NSB tubes.
6. Add 100 µl of $^{3}$H-testosterone tracer (Perkin Elmer-NEN, Boston, MA) to all tubes.
7. Mix the tubes gently by shaking and cover with foil. Incubate at 4°C for 16-20 hours.
8. Add 200 µl of charcoal solution to each tube except for the total tubes.
9. Vortex the tubes and incubate at 4°C for 15 minutes.
10. Centrifuge samples at 2000xg for 20 minutes.
11. Pour samples into scintillation tubes containing 5 ml of Ecolume biodegradeable scintillation cocktail (MP Biomedicals, Irvine, CA). Cap the tubes and shake them gently.
12. Count the samples for 1 minute each using a Beckman Beta counter.
13. Use AssayZap to calculate concentrations of unknowns in comparison to a known standard curve.

**Reagents:**

1. 0.01 M Phosphate Buffered Saline with 0.1% gel (PBSG)
   8.17 g Sodium Chloride
   0.856 g Sodium Phosphate monobasic ($NaH_2PO_4H_2O$)
   0.54 g Sodium Phosphate dibasic ($Na_2HPO_4$)
   3.72 g Ethylenediaminetetra acetic acid (EDTA)
   0.1 g Thimerosal powder (merthiolate)

   Dissolve above components in 900 ml of double-distilled water. Adjust pH to 7.0. Bring volume up to 1 liter with water. Add 1 g of gelatin and heat and stir on low for 2 hours. Cool and store at 4°C.

2. Charcoal
   3.125 g Charcoal
   0.3125 g Dextran Pharmacia T-70
   Add above components to 500 ml PBSG and stir at low speed overnight at 4°C to bring into solution. Stir charcoal for 30 minutes before each use.

# APPENDIX I

## *IN VITRO* TESTIS CULTURE

1. Collect stallion testicular parenchyma at the Veterinary Large Animal Medicine (VLAM) facilities and place in aseptic bags on ice.
2. Trim and weigh out 1 gram pieces of testicular parenchyma.
3. Transfer 1 gram of tissue into a 50-ml polypropylene tube containing 5 ml of DMEM media with Penicillin/Streptomycin.
4. Put each tube into a shaking water bath uncovered and when all tubes are in the water bath, cover the water bath with a lid as air tight as possible.
5. Incubate samples at 34°C with 95% $O_2$/5% $CO_2$ exposure for two hours.
6. Remove media and freeze at –20°C in appropriately labeled baseline 12X75 polypropylene tubes.
7. Add 5 ml treatments of either media alone, 10 units hCG, 100 units hCG, $10^{-5}$ M Forskolin or 1 mg/ml cyclic AMP to the testis tissue. Incubate for another two hours in the same conditions as before.
8. After two hours, the samples are divided into either 2-hr or 4-hr treatment groups. Media from the 2-hr treatment group is collected and frozen at –20°C. The testis samples are removed and frozen at –80°C in RNase-free tubes. Remove 200 µl of media from the 4-hr incubation samples and freeze. Continue the incubation for two more hours.
9. Remove the media from the 4-hr incubation samples and freeze at –20°C. Transfer the testis samples to RNase-free tubes and freeze at –80°C.

# APPENDIX J

## WESTERN BLOT PROCEDURE FOR StAR AND P450SCC PROTEINS

**Solutions:**

A. Acrylamide/Bis-acrylamide

87.6 g acrylamide (29.2 g/100 ml; Kodak cat# 5521)
2.4 g N,N'-bis-methylene-acrylamide (0.8 g/100 ml; Sigma cat# M-2022)
Make to 300 ml with distilled water. Filter and store at 4°C in the dark.

B. 1.5 M Tris-HCl, pH 8.8

27.23 g trizma base (Sigma cat# T-1503)
Mix in distilled water and adjust pH to 8.8 with 1N HCl. Make to 100 ml with distilled water.

C. 0.5 M Tris-HCl, pH 6.8

6 g trizma base (Sigma cat# T-1503)
Mix in distilled water and adjust pH to 6.8 with 1N HCl. Make to 100 ml with distilled water and store at 4°C.

D. 10% SDS

Dissolve 10 g sodium dodecyl sulfate (Sigma cat# L-4390) in distilled water with gentle stirring. Bring volume to 100 ml with distilled water.

E. Sample Buffer

| | |
|---|---|
| Distilled water | 4.0 ml |
| 0.5 M tris-HCl, pH 6.8 | 1.0 ml |
| glycerol (EM Science cat# GX0185-6) | 0.8 ml |
| 10% (w/v) SDS | 1.6 ml |
| 2-mercaptoethanol (Sigma M-3148) | 0.4 ml |
| 0.05% (w/v) bromophenol blue (JT Baker D293-01) | 0.2 ml |

Mix the above ingredients and store at room temperature. Dilute samples 1:4 with sample buffer and heat at 95°C for 10 minutes before loading gel.

F. 5X Electrode (Running) Buffer, pH 8.3

9 g trizma base (Sigma cat# T-1503)
43.2 g glycine (Sigma cat# G-7126)
3 g sodium dodecyl sulfate (Sigma cat# L-4390)

Mix and bring to 600 ml with distilled water. Store at 4°C. Warm to 37°C if precipitation occurs. Dilute 60 ml 5X stock buffer with 240 ml distilled water for use.

G. Lower Tris, pH 8.8

18.15 g trizma base (Sigma cat# T-1503)
4 ml 10% SDS
Mix in distilled water. Adjust pH to 8.8 and bring to 100 ml with distilled water. Filter solution.

H. Upper Tris, pH 6.8

6 g trizma base (Sigma cat# T-1503)
4 ml 10% SDS
Mix in distilled water. Adjust pH to 6.8 and bring volume to 100 ml with distilled water. Filter solution.

I. Tris-Sucrose-EDTA Buffer

0.607 g trizma base (Sigma cat# T-1503)
42.79 g sucrose (Sigma cat# S-0389)
0.145 g EDTA (Ethylenediaminetetra acetic acid; Sigma cat# E-5134)
Mix ingredients in distilled water. Adjust pH to 7.4 and bring volume to 500 ml in distilled water.

J. 10% Ammonium Persulfate

Mix 1.5 g ammonium persulfate (Sigma cat# A-9164) in 15 ml distilled water by inversion. Good for one month maximum, store at 4°C.

K. Mini Gel (12.5%; for 2 gels)

7.5 ml acrylamide/bisacrylamide
4.5 ml lower tris
6 ml distilled water
75 µl 10% ammonium persulfate
15 µl temed

L. Mini Gel Stacker (3%; for 2 gels)

450 µl acrylamide/bisacrylamide
1125 µl upper tris
2.88 ml distilled water
37.5 µl 10% ammonium persulfate
15 µl temed

M. Transfer Buffer

2.42 g trizma base (Sigma cat# T-1503)
11.24 g glycine (Sigma cat# G-7126)
100 ml methanol (Sigma cat# M-3641)
0.1 g sodium dodecyl sulfate (Sigma cat# L-4390)
Mix and bring to 1 liter with distilled water.

N. PBS + 0.25% TWEEN Buffer
8 g sodium chloride (NaCl; EM Science cat# 7710)
0.2 g potassium chloride (KCl; Fisher Scientific cat# P-217)
1.44 g sodium phosphate dibasic ($Na_2HPO_4$; Fisher Scientific cat# S-374)
0.24 g potassium phosphate monobasic ($KH_2PO_4$; EM Science cat# PX1565-1)
Mix and bring to 1 liter with distilled water. Add 2.5 ml TWEEN-20 (polyoxyethylene-sorbitan monolaurate; Sigma cat# P-1379).

O. Blocking Buffer (4%)
Mix 4 g carnation powdered milk in 100 ml PBS + Tween Buffer.

P. Stripping Buffer
2 g sodium dodecyl sulfate (Sigma cat# L-4390)
0.757 g trizma base (Sigma cat# T-1503)
700 µl 2-mercaptoethanol (Sigma cat# M-3148)
Mix in distilled water. Adjust pH to 6.8 and bring volume to 100 ml with distilled water.

Q. Strip Wash Buffer
0.12 g trizma base (Sigma cat# T-1503)
0.876 g sodium chloride (NaCl; EM Science cat# 7710)
Mix in distilled water. Adjust pH to 7.4 and bring volume to 100 ml with distilled water.

**Western Blot Procedures:**

**A. Protein Isolation**
1. Trim 200 mg of frozen tissue and place in Tris-Sucrose-EDTA buffer in a 50-ml tube (ex: 0.2 g in 2 ml buffer).
2. Homogenize samples for 30 seconds each two times.
3. Centrifuge the homogenized samples at 600xg for 15 minutes.
4. Remove the supernatant and transfer to a 2-ml microfuge tube.
5. Centrifuge at 12,000 rpm for 15 minutes.
6. Resuspend the pellet in 0.5 ml Tris-Sucrose-EDTA buffer.

**B. Protein Assay**
1. In a separate microfuge tube, dilute the samples 1:10 with water.
2. Dilute 4 µl BSA in 196 µl water and pipette 0, 2, 3, 4, 5, 7.5, 10, 12.5, 15, 17.5, and 20 µl in duplicate in a 96-well plate. (Concentrations = 0, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 µg)
3. Pipette 3 µl of each diluted sample in duplicate in the same 96-well plate.
4. Add 200 µl of Bradford Reagent diluted 1:5 in water.
5. Allow the samples to incubate at room temperature for 10 minutes.
6. Read the plate at Optical Density (OD) 562 to obtain absorbance values for each sample with a plate reader and calculate sample concentrations.

7. Centrifuge samples (from #6) at 12,000 rpm for 15 minutes.
8. Pour off supernatant and freeze samples at –80°C until ready to run gel.

**C. Gel Preparation**

1. Pour polyacrylamide gel according to recipe above pouring the lower portion without air bubbles and stopping at the bottom of where the comb will be. Overlay water on top of the gel while it polymerizes to keep the gel from drying out. Pour off water and dry with 3 mm whatman paper before pouring stacker gel on top. Stacker gel should be poured with the 10-well comb in place without air bubbles and allowed to polymerize.
2. Resuspend samples in sample loading buffer at a concentration of 4 µg/µl.
3. Resuspend lyophilized MA-10 marker (500 µg) in 500 µl sample loading buffer and use 5 µl per gel.
4. Load 5-7 µl of pre-stained molecular weight marker (cat# 161-0305; Bio-Rad, Hercules, CA).
5. Run the gel at 4°C in 1X Electrode Buffer for 30 minutes at 200 volts.
6. Remove the gel and notch at well 1.
7. Place the gel in cold Transfer Buffer for 5 minutes.
8. Cut two pieces of 3 mm Whatman paper to the same size as the sponge.
9. Cut the PVDF membrane (cat# 162-0184; Bio-Rad, Hercules, CA) to the same size as the gel.
10. Wet the membrane in methanol, soak it in water until saturated, then place the membrane in transfer buffer.
11. Set up the transfer apparatus using the following explanation (all items should be soaking in transfer buffer):

    YOU

    Plastic grid (clear side of mini-gel)
    Sponge pad
    3mm Whatman paper
    PVDF membrane
    Gel (back-side facing up)
    3mm Whatman paper
    Sponge pad
    Plastic grid (black side of mini-gel)

    LAB BENCH

    **Make sure there are no air bubbles between the gel, PVDF membrane and the Whatman paper.
12. Place the plastic grids in the transfer apparatus where the gel is on the black side and the membrane is on the red side. Fill the transfer apparatus with cold transfer buffer and a frozen ice pack in order to keep overheating from taking place.
13. Run at 100 volts for 2 hours. The transfer apparatus may have to sit in a bucket of ice to keep the buffer cool.

**D. Membrane Preparation**

1. Soak the membrane in Blocking Buffer while shaking at 4°C for 1 hour or overnight.
2. Make primary antibody in 2% milk solution at 1:1000 dilution.
3. Remove the blocking buffer and shake the membrane in primary antibody for 1 hour at room temperature (StAR antibody-Dr. Stocco, Texas Tech University, TX; P450scc antibody-Chemicon, Temecula, CA).
4. Remove the primary antibody solution and save it at 4°C (**can be used twice**).
5. Wash the membrane with PBS + Tween Buffer 3 times for 5 minutes each (shake).
6. Pour off wash buffer and shake the membrane in secondary antibody for 30 minutes at room temperature (cat# NA 934; Amersham Pharmacia, Piscataway, NJ; 1:15,000 dilution—3.3 µl in 50 ml 2% milk solution).
7. Remove the secondary antibody solution and save at 4°C (can be used twice).
8. Wash the membrane with PBS + Tween Buffer 2 times for 30 minutes each (shake).
9. Remove the membrane and expose protein side to chemiluminescence reagent (NEN cat# NEL-102) for 1 minute (5 ml of each mixed).
10. Place the membrane in sheet protector or saran™ wrap and expose to film immediately (Hyperfilm MP from Amersham Biosciences, Piscataway, NJ; cat# RPN 1678H).
11. Membranes can be stored moist at 4ºC in a Ziploc bag for 1 month.

**E. Stripping and Re-blotting Westerns**

1. Soak the membrane in strip buffer for 30 minutes at 70°C, shaking every 10 minutes.
2. Wash the membrane in wash buffer 2 times for 10 minutes each at room temperature (shake).
3. Rinse the membrane with PBS + Tween Buffer for 5 minutes.
4. Block the membrane with Blocking Buffer as before and proceed with antibody staining procedures above.

*If you are restaining with the same antibody, strip the membrane using 0.1 M Glycine, pH 2.6 for 5-10 minutes at room temperature. Rinse with PBS + Tween Buffer, block and restain membrane as before.

**If the membrane has dried out considerably, wet the membrane very quickly in methanol and then begin soaking in strip buffer.

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